UNIVERZITA KARLOVA V PRAZE

3. lékařská fakulta

Funkcia chromatín-remodelujúceho komplexu SWI/SNF v onkogenéze a progresii melanómových buniek

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Praha, 2013

DIZERTAČNÁ PRÁCA

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Názov dizertačnej práce: Funkcia chromatín-remodelujúceho komplexu SWI/SNF v onkogenéze a progresii melanómových buniek

Názov dizertačnej práce anglicky: Function of SWI/SNF chromatin remodeling complex in tumor initiation and progression of melanoma cells

Obor: Molekulární a buněčná biologie, genetika a virologie

Školiace pracovisko: Ústav lékařské biochemie a laboratorní diagnostiky, 1. LF Univerzity Karlovy v Praze a VFN

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V Prahe, 17. 4. 2013

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Poďakovanie

Rada by som úprimne poďakovala svojmu školiteľovi Doc. MUDr. Jiřímu Vachtenheimovi, CSc. za jeho odborné vedenie, cenné rady a čas, ktorý mi počas štúdia ochotne venoval. Vďaka za podporu a pomoc pri experimentálnej práci patrí aj mojim kolegom z ÚLBLD. V neposlednej rade ďakujem svojej milej rodine a priateľom za neustálu podporu a trpezlivosť, ktorú so mnou mali počas celého štúdia.

Použité skratky

ATP	adenosine triphosphate
Brg1	brahma-related gene 1
Brm	brahma
bHLH-LZ	basic helix-loop-helix-leucine zipper
CBP	CREB-binding protein
CREB	cAMP response element binding protein
CDK	Cyclin-dependent kinase
dn	dominant negative
HAT	histon acetyltransferase
HDAC	histon deacetylase
hGCN5	human general control nonderepressible 5
CHIP	chromatin immunoprecipitation
IGF1	insulin-like growth factor 1
INI1 (hSNF5)	interactor of integrase 1
IVT	*in vitro* transcription aand translation
IHC	immunohistochemistry
MITF	microphthalmia-associated transcription factor
MRT	malignant rhabdoid tumour
mTOR	mammalian target of rapamycin
NSCLC	non-small-cell lung carcinoma
OPN	osteopontin
raptor	regulatory associated protein of TOR
rictor	rapamycin-insensitive companion of mTOR
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	small interferring RNA
shRNA	small hairpin RNA
SWI/SNF	the switch in mating type/sucrose nonfermenting complex in yeast
PCAF	P300/CBP associated factor
PI3K	phosphoinositide 3-kinase
PKB	protein kinase B
RSV	Rous sarcoma virus
SOX10	SRY-box containing gene 10
TAD	transcription activation domain
TNF	tumour necrosis factor
TBX2	T-box transcription factor 2
TRP-1	tyrosinase-related protein 1
TRP-2	tyrosinase-related protein 2
Wt	wild type

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1. Súhrn

Čoraz častejšie je vznik nádorových ochorení spájaný so zmenami chromatínovej remodelácie. SWI/SNF chromatín-remodelujúce komplexy sa podieľajú na regulácii génovej expresie zmenou chromatínovej štruktúry a v závislosti na kontexte, môžu vystupovať ako transkripčné aktivátory alebo represory. Energiu pre remodeláciu poskytuje ATPázová podjednotka, ktorou môže byť buď proteín Brm (Brahma) alebo Brg1 (Brahma related-gene 1). Malígny melanóm je agresívny druh rakoviny, ktorý sa vyznačuje vysokou rezistenciou k súčasnej terapii. Kľúčové postavenie v biológii melanómu má transkripčný faktor MITF (microphthalmia-associated transcription factor), ktorý stojí v centre transkripčnej regulácie od embryonálneho vývoja normálnych melanocytov, ich diferenciácie, udržania identity a prežívania nielen normálnych, ale aj nádorových melanocytov. V našej práci sme zistili, že aktívny SWI/SNF komplex je nevyhnutný pre expresiu MITF. Tento komplex je taktiež dôležitý pre expresiu niektorých cieľových génov MITF. Prežívanie melanómových buniek je absolútne závislé na funkčnom komplexe SWI/SNF a bunky si zachovávajú vysokú expresiu jednotlivých zložiek. Primárne sa v regulácii MITF uplatňujú Brg1-obsahujúce subkomplexy, ktoré môžu byť po strate expresie Brg1 funkčne kompenzované subkomplexami s Brm. V každom prípade pre prežívanie a proliferáciu melanómových buniek musí byť zachovaná expresia aspoň jednej ATPázy. Štúdium Brg1-umlčaných melanómových buniek odhalilo dôležitosť SWI/SNF komplexu nielen z hľadiska manipulácie hladiny MITF, ale aj v regulácii ďalších proonkogénnych faktorov podporujúcich proliferáciu a prežívanie buniek nezávisle na transkripčnej osi MITF. Napriek tomu, že SWI/SNF komplex v prevažnej väčšine rakovín vystupuje ako tumorový supresor, v proliferácii a prežívaní melanómových buniek má naopak zásadnú pozitívnu úlohu. Podobne ako v prípade niektorých ďalších typov rakoviny (prostaty, žalúdka alebo hrubého čreva), SWI/SNF komplex (alebo aspoň niektorý z jeho proteínov, obzvlášť Brg1) pôsobí ako nádorový promótor. Inaktivácia komplexu môže z tohoto dôvodu predstavovať nádejný cieľ v terapii melanómu.

Summary

There is an increasing evidence that alterations in chromatin remodeling play an important role in tumorigenesis. The SWI/SNF chromatin remodeling complexes contribute to the regulation of gene expression by altering the local chromatin structure. Depending on the context, they can act as either transcriptional activators or repressors. All SWI/SNF subcomplexes contain one of two ATPase subunits, Brm (Brahma) or Brg1 (Brahma related gene 1), which provide the energy for remodeling. Malignant melanoma is an aggressive cancer and is known for its notorious resistance to conventional anticancer therapies. MITF (microphthalmia-associated transcription factor) plays an essential role in melanoma biology and is placed on the central crossroad in the regulation of melanocyte development, differentation, maintenance of lineage identity, and survival of both normal and malignant melanocytes. Our results show that the active SWI/SNF complex is strictly required for the expression of MITF. This complex is also required for expression of some transcriptional MITF targets. The survival of melanoma cells is absolutely dependent on functional SWI/SNF complex and all subunits of this complex are expressed at high levels in melanoma cell lines. Primarily, Brg1-containing subcomplexes are more important for MITF expression and melanoma cells proliferation, but after Brg1 loss its function could be compensated by Brm. Anyway, at least one ATPase must be expressed in order for melanoma cells to express MITF and proliferate. The study of Brg1-knockdowned melanoma cells has revealed the importance of SWI/SNF subcomplexes resulted from the manipulation of level of MITF. Furthermore, proliferation and survival of melanoma cells is increased by other SWI/SNF-dependent prosurvival and pro-oncogenic factors independently of the MITF axis. Although SWI/SNF complex is mostly regarded as a tumor supressor, it is presented here its crucial positive role in melanoma proliferation and survival. Similarly as in the case of some other cancer types (prostate cancer, gastric and colorectal cancer), SWI/SNF complex, or at least some of its components (mostly Brg1) seems to be a tumor promoter for melanoma. Thus, inactivation of its activity may be a promissing target in melanoma therapy.

2. Úvod a literárny prehľad

Nádorová transformácia je proces komplexnej akumulácie genetických a epigenetických zmien, ktorého výsledkom je nekontrolovaný rast a deregulácia kritických dejov ako je bunkový cyklus, apoptóza a DNA reparačné mechanizmy. Keďže priebeh a postup nádorovej transformácie je v škále bunkových typov značne odlišný, je neustále predmetom intenzívneho skúmania. V posledných rokoch bolo kľúčovým poznatkom definovanie mechanizmu líniovej závislosti (lineage addiction), ktorý predstavuje dereguláciu expresie špecifických génov riadiacich normálny líniovo-špecifický vývoj. Aktuálne je pozornosť sústredená aj na ďalšiu skupinu génov, ktoré sú v nádoroch často mutované, a to gény zohrávajúce úlohu v regulácii štruktúry chromatínu. V dôsledku epigenetických zmien má chromatín nádorových buniek oproti normálnym bunkám napríklad odlišný status metylácie DNA a histónových modifikácii. Je nepochybné, že spolu s genetickými zmenami epigenetické zmeny prispievajú k onkogenéze a dôležitou výzvou je rozpoznať tento mechanizmus. V predkladanej práci sa zameriavam na štúdium funkcie chromatín-remodelujúceho komplexu SWI/SNF v melanomových bunkových líniách. Melanóm by mohol byť v prípade včasnej diagnostiky vďaka svojej dostupnosti jedným z najlepšie liečiteľných nádorov, avšak metastatický melanóm disponuje zlou prognózou a obrovskou rezistenciou k súčasnej terapii.

Chromatínová štruktúra je všeobecne regulovaná dvoma základnými skupinami multiproteínových komplexov. Prvou skupinou sú komplexy, ktoré kovalentne modifikujú histónové chvostíky (acetyláciou, fosforyláciou, metyláciou) a druhou skupinou sú komplexy remodelujúce nukleozómovú štruktúru. Obe formy, či už posttranslačné modifikácie alebo remodelácia chromatínu sa podieľajú na regulácii génovej expresie tým, že umožňujú prístup faktorom viažucim sa na DNA (Khorasanizadeh, 2004).

2.1. SWI/SNF komplex a jeho zloženie

Najlepšie charakterizovaným chromatín-remodelujúcim komplexom je SWI/SNF (the switch in mating type/sucrose nonfermenting complex in yeast). Tento evolučne vysoko konzervatívny komplex bol prvýkrát izolovaný z kvasiniek začiatkom osemdesiatych rokov minulého storočia pri štúdiu génov ovplyvňujúcich prepínanie párovacích typov kvasiniek (switch (SWI) mutants) a génov dôležitých v metabolizme sacharózy (sucrosenon-fermenting (SNF) mutants) v kvasinkách *Sacharomyces cerevisiae* (Wilson and Roberts, 2011). Má

dôležitú úlohu v mnohých bunkových procesoch ako je diferenciácia, proliferácia, replikácia DNA, či oprava poškodenej DNA. Jeho nefunkčnosť je spájaná s malígnou transformáciou a je považovaný za tumorový supresor.

SWI/SNF komplex je schopný meniť lokálnu štruktúru chromatínu, čím sprístupňuje špecifické sekvencie transkripčným faktorom. K zmene štruktúry alebo pozície nukleozómov využíva energiu z hydrolýzy ATP. Za túto funkciu je v komplexe zodpovedná jeho enzýmová podjednotka s ATPázovou aktivitou (Muchardt a Yaniv, 2001).

Ľudský SWI/SNF komplex má veľkost 2MDa a pozostáva z 9 - 12 proteínov. Napriek tomu, že jeho presné podjednotkové zloženie môže čiastočne kolísať, rozlišujeme dve základné skupiny SWI/SNF komplexov, BAF a PBAF. Obe skupiny obsahujú ATPázovú podjednotku, ktorou môže byť buď **Brm** (SMARCA2, brahma, BAF190B) alebo **Brg1** (SMARCA4, brahma-related gene 1, BAF190A) a variabilnú kombináciu tzv. BAF faktorov (Brm and Brg1 associated factors). BAF faktory vyskytujúce sa v oboch skupinách komplexov sú BAF47/INI1/SNF5 (SMARCB1), BAF53A-B (ACTL6A-B), BAF57 (SMARCE1), BAF60A-B (SMARCD1-2), BAF155 (SMARCC1), BAF170 (SMARCC2), a aktín (ACTB). PBAF komplexy obsahujú ďalšie špecifické podjednotky BAF180 (PBRM1, známe ako polybromo) a BAF200 (ARID2, polybromo-associated factor). BAF komplexy obsahujú špecifické podjednotky BAF250A a BAF250B (ARID1A a ARIDB) (zhrnuté v Reisman a spol., 2009; Wilson and Roberts, 2011).

2.2. Enzýmové podjednotky Brg1 a Brm

Ako už bolo spomenuté, enzýmovú funkciu v SWI/SNF komplexe plnia proteíny Brm a Brg1. V konkrétnom type SWI/SNF komplexu je zastúpený vždy len jeden z nich, pričom BAF komplexy obsahujú buď Brm alebo Brg1, ale PBAF komplexy obsahujú vždy len Brg1. Proteíny Brg1 a Brm sú veľmi podobné. U ľudí sú ich sekvencie na 74% identické, z čoho vyplývajú ich podobné enzymatické vlastnosti. Napriek tomu, že sú vo funkciách čiastočne zastupiteľné, počas proliferácie a diferenciácie prednostne interagujú s rozdielnymi transkripčnými faktormi. Brg1 sa viaže na proteíny obsahujúce motív zinkových prstov a Brm sa viaže na ankyrinové repetície (Kadam a Emerson, 2003). Substrátová špecificita jednotlivých typov SWI/SNF komplexu je teda závislá na prítomnosti konkrétnej ATPázy, ale ďalej je ovplyvnená kombináciou nekatalytických podjednotiek (jednotlivých BAF faktorov), ktoré priamou väzbou k doménam špecifických transkripčných faktorov zabezpečujú selektívnu remodeláciu chromatínu (Nagl a spol., 2007; Emerson, 2002). Vdôsledku toho

Brg1- a Brm-obsahujúce SWI/SNF komplexy môžu regulovať expresiu odlišných génov. Ich odlišné funkčné uplatnenie v bunke môže byť ďalej spôsobené primárnym rozdielom v expresii Brm a Brg1 v priestore a čase (Strobeck a spol., 2002). Inaktivácia Brg1 génu je v skorých štádiach embryonálneho vývinu letálna a embryá zomierajú pred štádiom implantácie. Hoci blastocyty s Brg1 nulovou mutáciou sa zdajú byť zjavne morfologicky normálne, nie sú schopné vykĺznuť z ich *zona pellucida* a ďalej proliferovať. Väčšina Brg1 heterozygotov prežívajú a sú plodní, ale náchylní k neopláziám. 15-30 % Brg1 myších heterozygotov postihnujú exencefálie a v 3 z 20 Brg1 heterozygotných myší sa vyvinuli vo veku 16 mesiacov rozsiahle kožné nádory na chrbte a v oblasti slabín (Bultman a spol., 2000). Na rozdiel od Brg1, po inaktivácii mBrm lokusu pomocou homológnej rekombinácie sú mutantné myši postrádajúce Brm životaschopné, avšak s hmotnosťou o 10-15% väčšou ako má divoký typ. Vo viacerých orgánoch (mozog, pečeň, slezina, obličky, pľúca, srdce) majú prítomnú zvýšenú hladina proteínu Brg1, ktorá pravdepodobne kompenzuje funkciu neprítomného Brm (Reyes a spol., 1998). Brg1 je konštantne exprimovaný v proliferujúcich alebo obnovujúcich sa bunkách a Brm je exprimovaný prevažne v mozgu, pečeni, fibromuskulárnej stroma a endotelových bunkových typoch, ktoré stabilne nepodliehajú proliferácii alebo samoobnove. (Reisman a spol., 2005)

Okrem synergického účinku môžu mať proteíny Brg1 a Brm účinok aj antagonistický. Príkladom je rozdielna úloha Brg1- a Brm-obsahujúcich SWI/SNF komplexov v regulácii transkripčnej aktivity androidného receptora na jeho cieľových promótoroch (Marshall a spol., 2003), či v p53-závislej regulácii p21 a MDM2 (Xu a spol., 2007). Ďalším príkladom protichodného účinku je úloha v diferenciácii osteoblastov, kde Brg1 pôsobí ako pozitívny regulátor a Brm má represívnu funkciu (Flowers a spol., 2009).

2.3. Biologické funkcie SWI/SNF komplexu

2.3.1. Úloha SWI/SNF komplexu v diferenciácii

SWI/SNF komplex má dôležitú úlohu v regulácii rovnováhy medzi diferenciáciou a proliferáciou. Proces remodelácie chromatínu je neoddeliteľnou súčasťou s diferenciáciou spojených ďalších procesov ako bunkový cyklus či replikácia. Úloha SWI/SNF komplexu v diferenciácii bola popísaná v mnohých bunkových líniách a môže byť buď bunkovo- alebo génovo-špecifická (Simone, 2006; de la Serna a spol., 2006). Je známe, že Myo-D, kritický "master" regulátor mesenchymálnych buniek, potrebuje aktivitu SWI/SNF komplexu. Dominantne negatívne mutanty Brg1 a Brm (dn Brg1 a dn Brm) inhibujú indukciu svalovej

diferenciácie sprostredkovanej proteínom Myo-D (de la Serna a spol., 2001) a dn Brg1 blokuje myeloidnú diferenciáciu 32Dcl3 buniek (Vradii a spol., 2006). Ablácia Brg1 pôsobí selektívne počas tvorby pokožky myší. Ukázalo sa, že strata Brg1 ovplyvnila terminálnu diferenciáciu keratinocytov, a tiež mala za následok vývojové defekty dolných končatín myší (Indra a spol., 2005). Brg1 reguluje aj diferenciáciu srdcových buniek prostredníctvom interakcie s histón-acetyltransferázami (HAT) a poly(ADP ribóza) polymerázou (PARP) a je dôležitý pre udržanie kardiomyocytov v diferenciačnom štádiu (Hang a spol., 2010). Ďalej bola popísaná úloha Brg1 v spermatogenéze, pričom delécia Brg1 v myšej zárodočnej línii úplne zastavila spermatogenézu v midpachytene štádiu (Wang a spol., 2012). V neurálnej diferenciácii je Brg1 podstatný pre proneurálnu funkciu bHLH transkripčných faktorov Ngn1 a NeuroD (Seo a spol., 2005), a taktiež sa zúčastňuje ako koaktivátor transkripčnej aktivácie melanocytovo-špecifických génov (de la Serna a spol., 2006; Keenen a spol., 2010).

2.3.2. SWI/SNF komplex a proliferácia

SWI/SNF komplex je spájaný s reguláciou bunkovej proliferácie prostredníctvom niekoľkých signálnych dráh, ale asi najlepšie je dokumentované prepojenie faktorov SWI/SNF komplexu s regulátormi bunkového cyklu cez interakciu Brg1 a Brm s Retinoblastoma proteínom pRb (Wilson a Roberts, 2011). Tumor-supresorový proteín pRb má fundamentálnu úlohu pre prechod z G1 do S fázy bunkového cyklu a tento kontrolný bod reguluje prostredníctvom interakcie s transkripčnými faktormi z E2F rodiny. V neskorej G1 fáze, keď je pRb hyperfosforylovaný prostredníctvom komplexu D-cyklín/cyklíndependentná kináza 4/6, sú E2F faktory uvoľnené a následne je aktivovaná transkripcia ich cieľových génov dôležitých pre S fázu bunkového cyklu. Brm a Brg1 interagujú s proteínmi pRb rodiny (Strober a spol., 1996) a kooperujú v indukcii zastavenia bunkového cyklu (Reisman a spol., 2009). Trouche a spol. (1997) zistili úlohu Brm v pRb-sprostredkovanej inhibícii transaktivácie transkripčných faktorov E2F. Na druhej strane, v skorej G1 fáze sprístupňuje SWI/SNF komplex E2F faktory svojim cieľovým promótorom, a teda sa podieľa na E2F aktivácii. Nie je úplne jasné, od čoho závisí aktivačný alebo represívny efekt SWI/SNF komplexu, tento účinok sa však zdá byť závislý na acetylačnom statuse histónových chvostíkov chromatínu (Muchardt a Yaniv, 2001).

SWI/SNF sa ďalej podieľa na regulácii génu primárnej odpovede Myc, ktorého proteinový produkt hrá kľúčovú úlohu v regulácii proliferácie a apoptózy a je často "overexprimovaný" v rakovinových bunkách. Účinok SWI/SNF môže byť protichodný v závislosti na podjednotkovom zložení komplexu. Počas diferenciácie ARIDA-obsahujúce

SWI/SNF komplexy reprimujú expresiu Myc a ARIDB-obsahujúce komplexy naopak výrazne podporujú transkripciu génu Myc (Wilson a Roberts, 2011). Už dávnejšie bolo zistené, že podjednotka INI1 sa priamo viaže s Myc a kooperuje v aktivácii cieľových génov Myc *in vitro* (Cheng a spol., 1999). Z uvedených príkladov vyplýva, že SWI/SNF komplex sa podieľa na regulácii faktorov ovplyvňujúcich proliferáciu.

2.3.3. Ďalšie biologické funkcie SWI/SNF komplexu

DNA replikácia a oprava poškodenej DNA sú ďalšie bunkové procesy vyžadujúce sprístupnenie DNA prostredníctvom chromatín-remodelujúcich komplexov. SWI/SNF chromatín-remodelujúce faktory zohrávajú úlohu na rôznych úrovniach nukleotidovej vyštepovacej opravy a opravy dvojreťazcových zlomov (Osley a spol., 2007). Niekoľko proteínov zúčastňujúcich sa procesov opravy DNA, napr. p53 a BRCA1, je asociovaných so SWI/SNF a sú na ňom funkčne závislé. Transkripčný faktor BRCA1 je tumor-supresorový proteín a mimo iných funkcií je koaktivátorom transkripcie aktivovanej prostredníctvom p53. BRCA1 sa priamo viaže s Brg1 a jeho p53-stimulačná funkcia je inhibovateľná dn Brg1 (Bochar a spol., 2000).

2.4. SWI/SNF komplex a nádory

Keďže SWI/SNF sa podieľa na regulácii mnohých procesov v bunke, nie je prekvapivé, že nefunkčný komplex prispieva k malígnej transformácii. Mnoho vedeckých prác popisuje jeho tumor-supresorovú funkciu, ktorú naznačila už spomínaná spojitosť napríklad s tumor-supresorovým proteínom pRb. SWI/SNF komplex interaguje tiež s tumor-supresorovým proteínom p53 (Lee a spol., 2002), ß-katenínom (Barker a spol., 2001), a tiež s produktami proto-onkogénov c-fos, c-jun (Ito a spol., 2001) a c-myc (Cheng a spol., 1999). Vo viacerých typoch primárnych nádorov a nádorových bunkových línií boli popísané mutácie podjednotiek SWI/SNF komplexu.

Jasne definovaným tumorovým supresorom je INI1. Proteín INI1 je jednou z najmenších podjednotiek SWI/SNF komplexu a je bialelicky inaktivovaný takmer u 100% malígnych rhabdoidných tumorov (MRT) (Versteege a spol., 1998). MRT sú vzácne agresívne nádory vznikajúce v mozgu, obličkách a iných mäkkých tkanivách s veľmi zlou prognózou postihujúce hlavne deti v rannom detstve. INI1 je tiež mutovaný v prípade karcinómu choroidného plexu, meduloblastómu, primitívnych neuroektodermálnych nádorov a primárnych rhabdomyosarkómov. Analýzou množstva rhabdoidných nádorových

bunkových línií sa zistilo, že INI1 je inaktivovaný hlavne homozygotnými deléciami, nonsense, missence a posunovými mutáciami (Biegel a spol., 2002; Versteege a spol., 1998; DeCristofaro a spol., 1999). V myších modeloch delécia INI1 spôsobuje letalitu v ranných fázach embryonálneho vývoja. Heterozygotné INI1-mutované myši boli viabilné, avšak v dôsledku straty heterozygozity u 3-35% vznikli v dospelosti nádory (Muchardt a Yaniv, 2001). Viac informácii o INI1 je v Prílohe 6, str. 115.

Druhým tumor-supresorovým proteínom SWI/SNF komplexu je Brg1. 30-40% pľúcnych nádorových línií postráda expresiu Brg1 alebo Brm. 15-50% ľudských prímárnych nemalobunkových nádorov pľúc (NSCLC) postráda expresiu Brg1 alebo Brm. V 10-20% vzoriek nádorov obličiek, hrubého čreva, prsníka, krku, vaječníkov, pankreasu a melanómu nebola detekovaná expresia Brg1 alebo Brm. 35% NSCLC línií nesie homozygotné mutácie Brg1 a Brg1 je mutovaný aj v ďalších bunkových líniách, napr. karcinómu pľúc, prostaty, prsníka, pankreasu (Reisman a spol., 2009; Wilson a Roberts, 2011; Wong a spol., 2000). Brg1 priamo interaguje s tumor-supresorovým proteínom BRCA1 a dn Brg1 má za následok kompletnú inhibíciu p53-sprostredkovanej transkripcie BRCA1 v bunkách karcinómu prsníka (Bochar a spol., 2000). Okrem toho, že interaguje s pRb a zvyšuje jeho transkripčnú represorovú aktivitu, pôsobí tiež “upstream” nad pRb, a to tak, že reprimuje expresiu cyklínu D (Rao a spol., 2008) a “upreguluje” expresiu CDK inhibítorov p21, p15 a p16 (Kang a spol., 2004; Hendricks a spol., 2004). Je zaujímavé, že mutácie Brg1 sú oveľa častejšie v nádorových líniách ako v primárnych nádoroch, v ktorých bola vo väčšine prípadov detekovaná strata heterozygozity v Brg1 lokuse (Reisman a spol., 2009; Wilson a Roberts, 2011).

Na rozdiel od Brg1, Brm je mutovaný zriedka a oveľa častejšie je pozorovaná strata jeho expresie, ktorá je potlačená epigenetickým mechanizmom (Glaros a spol., 2007; Reisman a spol., 2009). Liu a spol. (2011) objavili dva polymorfizmy v promótorovej oblasti Brm génu, v ktorých je väzobné miesto pre transkripčný faktor MEF2 (myocyte enhancer factor-2). MEF2 rekrutuje HAT, ktoré sú zodpovedné za umlčanie expresie Brm.

V posledných rokoch začína byť diskutovaná okrem tumor-supresorovej funkcie SWI/SNF aj jeho proonkogénna úloha v nádorovej progresii. Imunochemicky bola detekovaná zvýšená expresia Brg1, nie však Brm, v nádoroch hrubého čreva. Knockdown Brg1 v bunkovej línii kolorektálneho karcinómu mal za následok zníženú proliferáciu, zvýšenú expresiu tumor-supresorového proteínu PTEN nasledovanú inhibíciou PI3K-Akt signálnej dráhy a poklesom expresie cyklínu D1 (Watanabe a spol., 2011). Zvýšená hladina expresie Brg1 bola pozorovaná imunohistochemicky v prípadoch rakoviny žalúdka (Sentani a

spol., 2001), prostaty (Sun a spol., 2007), či melanómu (Lin a spol., 2010). Uvedené poznatky naznačujú odlišný líniovo- a/alebo tkanivovo-závislý biologický význam SWI/SNF komplexu v patogenéze ľudských nádorov.

2.5. Malígny melanóm a MITF

Zo skupiny kožných nádorov predstavuje malígny melanóm najnebezpečnejšiu formu. Vzniká z melanocytov, špeciálnych pigmentových buniek, ktoré pochádzajú z progenitorových buniek neurálnej lišty migrujúcich počas embryonálneho vývoja do pokožky. Kožné melanocyty sú lokalizované v bazálnej vrstve pokožky a vo vlasových folikulách a ich funkciou je ochrana pokožky pred UV žiarením. Pôsobením UV žiarenia keratinocyty produkujú faktory stimulujúce produkciu melanínu v melanozómoch, špeciálnych organelách melanocytov. Melanozómy sú následne transportované do susedných keratinocytov, kde melanín poskytuje ochranu pred UV-sprostredkovaným poškodením DNA. Nadmerná produkcia melanocytov uvoľnená z kontroly keratinocytov umožňuje vznik melanocytických lézií névusov. Névusy sú vo všeobecnosti benígne, ale môžu sa stať prekurzorom malígneho melanómu. Prechod od névusu k melanómu zahrňujúci niekoľko fáz zmien nemusí byť vždy pravidlom a metastatický melanóm sa môže vyvinúť priamo z izolovaných melanocytov alebo névusu (Gray-Schopfer a spol., 2007; Goding, 2000; Vachtenheim a Borovanský, 2010).

2.5.1. Signálne dráhy v melanómoch

Až 90% ľudských melanómov má hyperaktivovanú Ras/Raf/MEK/ERK signálnu dráhu (Cohen a spol., 2002). Mutácie BRAF, jedného z komponentov tejto signálnej dráhy, sú prítomné v 50-70% melanómov. Najčastejšia mutácia je substitúcia V600E na pozícii 600 spôsobujúca zámenu kyseliny glutámovej za valín (Davies a spol., 2002). Pozoruhodné je, že BRAF je mutovaný až v 80% benígnych névusov (Pollock a spol., 2003), z ktorých len vzácne vznikne melanóm. Samotný onkogénny (V600E)BRAF nie je postačujúci pre malígnu transformáciu, ale naopak, je zodpovedný za $p16^{INK4A}$-sprostredkovanú indukciu senescencie v névusoch nazývanej tiež onkogénom-indukovaná senescencia (oncogene-induced senescence) (Gray-Schopfer a spol., 2006). V 15-30% melanómov je mutovaný ďalší komponent Ras/Raf/MEK/ERK signálnej dráhy NRAS. NRAS je produktom jedného z troch ras génov a najčastejšou mutáciou je substitúcia Q61L leucínu za glutamín v pozícii 61 (Davies a spol., 2002).

Ďalšou často deregulovanou signálnou dráhou v melanómoch je dráha fosfoinositid-3-OH kinázy (PI3K), PI3K/Akt. "Downstream" efektor tejto dráhy, tumor-supresorový proteín PTEN je inaktivovaný v 5-20% melanómov v pokročilých štádiách. Proteín kináza B (PKB známa tiež ako Akt), ďalší efektor PI3K dráhy je overexprimovaná asi v 60% melanómov (Gray-Schopfer a spol., 2007). Je nutné spomenúť, že v malígnych melanómch je vysoko aktivovaná taktiež ß-katenínová signalizácia (Widlund a spol., 2002).

Podobne ako v prípade mnohých iných neoplázií, fyziologická diferenciácia melanocytov a vývoj malígneho melanómu zdieľajú spoločné signálne dráhy (Busca a Balotti, 2000). Centrálny význam v biológii melanocytov predstavuje transkripčný faktor MITF (Microphthalmia-associated transcription factor).

2.5.2. MITF

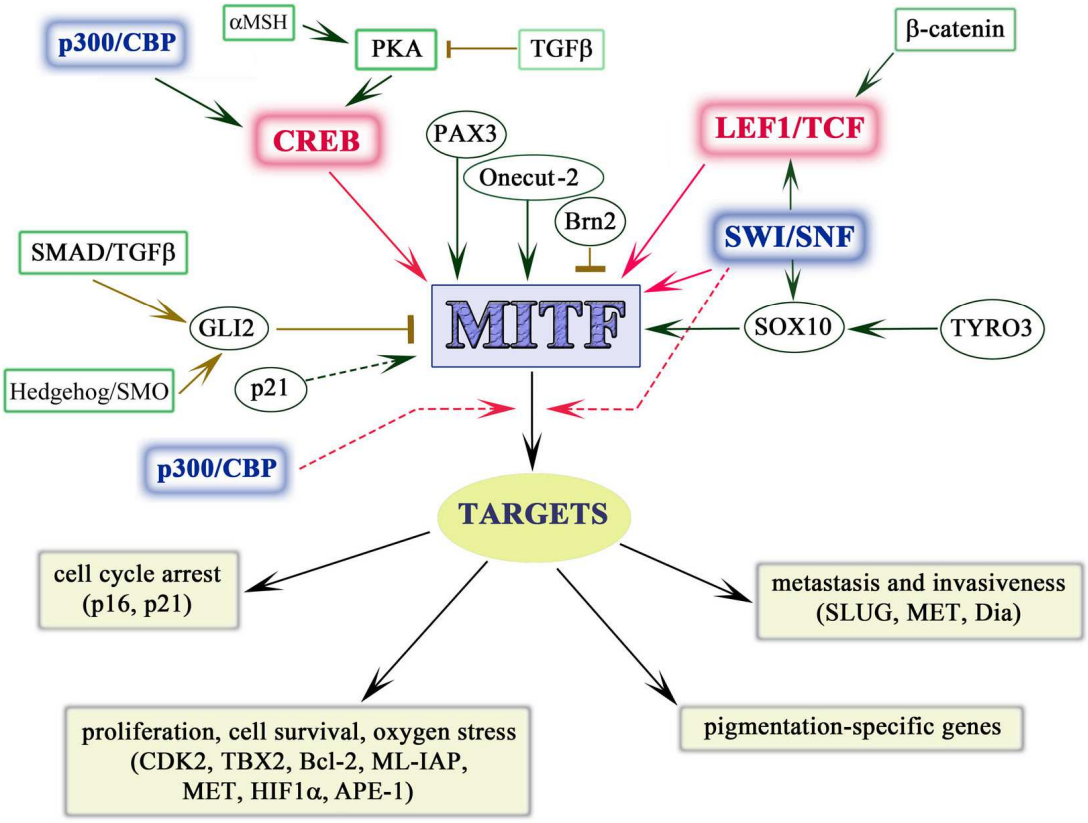
MITF má kritickú úlohu pre celý vývoj melanocytovej línie od embryonálnych melanocytov, pre prežívanie melanocytov ako aj melanómových buniek. Existuje vo viacerých zostrihových izoformách (A, B, C, D, H, M, mc), z ktorých každá je exprimovaná z vlastného, tkanivovo-špecifického promótora (Amae a spol., 1998; Udono a spol., 2000; Shibahara a spol., 2001; Takemoto a spol., 2002). Jednotlivé izoformy sa odlišujú prvým exónom, ostatné exóny sú spoločné. Pre melanocyty je špecifická forma MITF-M (Fuse a spol., 1996) (v tejto práci reprezentovaná označením MITF). MITF patrí do skupiny bHLH-LZ (bázický helix-slučka-helix leucínový zips) transkripčných faktorov. V súčasnosti je známych viac ako 40 cieľových génov MITF, ktorých účinky sú častokrát antagonistické (napr. CDK2 a CDKN1A) (Cheli a spol., 2010). MITF aktivuje transkripciu širokej palety génov zúčastňujúcich sa melanocytovej diferenciácie (DIA, SILVER, OA1) a syntézy pigmentu melanínu (napr. tyrozináza, TRP1, TRP2) (Vachtenheim a Borovanský, 2010). Je transkripčným regulátorom mnohých génov kontrolujúcich melanocytovú proliferáciu a prežívanie, napríklad pre CDK2 (Du a spol., 2004) a TBX2 (T-box transcription factor 2), ktorý je dôležitý v zachovaní proliferácie a potlačení senescencie melanómových buniek prostredníctvom "downregulácie" p21 (Prince a spol., 2004). Ďalšími dôležitými transkripčnými cieľmi sú antiapoptotický faktor BCL2 (McGill a spol., 2002), či faktor HIF1α (Busca a spol., 2005) zohrávajúci kľúčovú úlohu v transkripčnej odpovedi na hypoxiu. MITF transkripčne aktivuje c-MET (McGill a spol., 2006), ktorý je vo vysokej miere exprimovaný v ľudských melanómoch a spájaný s jeho metastatickým potenciálom. Na druhej strane je MITF transkripčným aktivátorom génov s protichodnou funkciou, napríklad inhibítorov bunkového cyklu p21 a p16 (Carreira a spol., 2005; Loercher AE a spol., 2005).

Táto aktivita MITF predstavuje významnejšiu úlohu pravdepodobne v normálnych melanocytoch pri kontrole G1 fázy bunkového cyklu. V melánómoch je p16 často inaktivovaný mutáciami alebo metyláciou promótora a p21 prostredníctvom pôsobenia TBX2 (Prince a spol., 2004; Vance a spol., 2005; Carreira a spol., 2006). Na Obr. 1 je transkripčná regulačná sieť, ktorej centrálnym faktorom je MITF. Podrobnejšie spracovanie regulácie expresie MITF a jeho transkripčnej aktivity je v Prílohe 7 (str. 133).

Známe koaktivátory MITF sú proteíny p300/CBP (CREB binding protein). Proteíny P300/CBP sú globálne transkripčné koaktivátory, ktoré slúžia ako kofaktory pre mnohé odlišné transkripčné faktory. Viažu sa na MITF a stimulujú transkripciu z citlivých promótor-reportérov (Sato a spol, 1997; Price a spol., 1998). p300/CBP patria do skupiny histón-acetyltransferáz a sú schopné acetylovať históny a nehistónové proteíny, z ktorých mnohé sú transkripčné faktory (Goodman a Smolik, 2000). Acetylácia N-terminálnych lyzínových zvyškov histónových chvostíkov neutralizuje ich záporný náboj a oslabuje interakciu medzi histónmi a DNA. Popri chromatínovej remodelácii patrí k epigenetickým mechanizmom, ktoré ovplyvňujú transkripčnú aktivitu na promótorových oblastiach génov (Khorasanizadeh, 2004). Je pravdepodobné, že MITF rekrutuje p300/CBP k cieľovým promótorom a sám je nimi acetylovaný (naše nepublikované výsledky).

Funkcia ďalších HAT ako koaktivátorov MITF nie je jasná. Študovali sme úlohu dvoch HAT, PCAF (P300/CBP associated factor) a hGCN5 (human general control nonderepressible 5) v regulácii aktivity MITF a výsledky sú spracované v Prílohe 8 (str.144). Táto príloha predstavuje publikáciu v príprave a vyžaduje si dokončenie.

Ektopický MITF spolu s (V600E)BRAF bol postačujúci k transformácii primárnych ľudských melanocytov a na základe nevyhnutnosti expresie MITF pre prežívanie melanómových buniek je MITF považovaný za tzv. líniovo-špecifický onkogén (lineage addiction oncogene) (Garraway a spol., 2005). Takýto faktor je za normálnych podmienok postačujúci pre udržanie identity bunkovej línie, ale po nádorovej transformácii je nevyhnutný aj pre prežívanie nádorových buniek, obvykle prekonaním apoptotických stimulov a podporou proliferácie. King a spol. (2001) vo svojej práci zistili, že expresia MITF je zachovaná v 85% melanómov a MITF gén bol amplifikovaný v 20% metastatických melanómov. Cronin a spol. (2009) analyzovali 50 metastatických melanómových línií a zistili, že 20% prípadov má porušenú MITF cestu. Citlivosť melanómových línií na represiu MITF sa môže líšiť a kombinovaná represia MITF a (V600E)BRAF prostredníctvom shRNA umocnila inhibičný účinok na rast melanómových buniek dokonca v prípadoch MITF-rezistentnejších bunkových línií (Kido a spol., 2009).



Obr. 1 Transkripčná sieť MITF v melanómoch

Na obrázku sú znázornené upstream MITF aktivátory (pravdepodobne najdôležitejšie sú červené), modré sú znázornené koaktivátory. Downstream transkripčné ciele MITF sú znázornené ako "targets". Prerušovaná čiara značí, že ňou indikovaná epigenetická regulácia nemusí platiť pre všetky ciele MITF.

Enzýmy SWI/SNF komplexu sa podieľajú na regulácii niekoľkých špecifických melanocytových génov. MITF rekrutuje SWI/SNF komplex k ich promótorom, ktorý následne môže remodelovať lokálnu chromatínovú štruktúru. Brg1 sa viaže na niekoľko promótorov závislých na transkripčnej aktivite MITF, napríklad promótor pre livin (ML-IAP), potenciálny antiapoptotický faktor, ktorý je silno exprimovaný v melanómoch (Dynek a spol., 2008). Brg1 sa ďalej viaže na promótory génov kódujúcich melanínovú syntézu (tyrozináza, trp1 a trp2), promótor génu rab27A, ktorého produkt reguluje transport melanozómov (Chiaverini a spol., 2008) a promótor antiproliferačného génu p21. Na druhej strane sa neviaže na promótory génov cdk2, bcl2 alebo tbx2 (Keenen a spol., 2010; Saladi a spol., 2010a). V prílohe 6 (str. 115) je podrobnejšie spracovaná úloha SWI/SNF-chromatínovej remodelácie v normálnych a nádorových melanocytoch.

Až v 80% prípadov benígnych névusov je mutovaný BRAF. Mutácia (V600E)BRAF, ako už bolo spomenuté, indukuje v normálnych melanocytoch p16-sprostredkovanú senescenciu a prekonanie senescenčného programu je pravdepodobne spôsobené inaktiváciou p16/pRb dráhy (Gray-Schopfer a spol., 2007). Brg1 priamo interaguje s p16 v melanómových bunkách, ale nebola zistená jeho úloha v p16-indukovanej senescencii (Becker a spol., 2009). Avšak Brg1 interaguje taktiež s MITF, ktorý je aktivátorom expresie p16 a ß-katenínom, ktorý je represorom expresie p16 (Saladi a spol., 2010a). ß-katenínová dráha je často deregulovaná
v melanómoch (Widlund a spol., 2002) a efektor tejto dráhy LEF1, ako už bolo spomenuté, je koaktivátorom transkripčného faktora MITF. Dôkladnejšie objasnenie funkcie SWI/SNF komplexu v kontexte transkripčnej regulácie MITF, ktorým sa zaoberáme v našej práci (Príloha 1, str. 51 a Príloha 2, str. 57), by mohlo prispieť k návrhu efektívneho terapeutického postupu v protinádorovej génovej terapii melanómu.

2.6. p21 a MITF

Významný inhibítor cyklín-dependentných kináz p21 sa podieľa na regulácii bunkového cyklu prostredníctvom priamej väzby na cyklín-CDK komplexy. Ako jediný z inhibítorov bunkového cyklu pôsobí univerzálne a inhibuje aktivitu všetkých cyklín-CDK komplexov. p21 je efektor tumor-supresorového proteínu p53, ktorý sa aktivuje hlavne pri poškodení DNA a jeho zvýšená expresia bola tiež detekovaná v senescentných bunkách (Harper a spol., 1995; El-Deiry a spol., 1993). Prostredníctvom väzby na CDK-komplexy inhibuje fosforyláciu tumor-supresorového proteinu pRb, následkom čoho je blokovaná E2F-riadená transkripcia (viz vyššie kapitola 2.3.2.). Okrem tejto nepriamej regulácie sa podieľa na transkripčnej regulácii aj priamo prostredníctvom interakcie s transkripčnými faktormi. p21 sa viaže napr. na spomínané transkripčné faktory E2F1 (Delavaine a La Thangue, 1999), onkoproteín c-Myc (Kitaura a spol., 2000) a STAT3 (Coquret a Guscan, 2000) a inhibuje ich aktivititu. Bola popísaná aj funkcia p21 ako transkripčného koaktivátora, napr. v prípade estrogénneho receptora (Poole a spol., 2004). Ďalšou jeho funkciou, ktorá môže byť nezávislá na funkcii inhibovať cyklín-CDK komplexy, je jeho antiapoptotická aktivita. p21 sa viaže na prokaspázu 3 a bráni vzniku aktivovanej kaspázy 3. Tento účinok je balancovaný tým, že p21 je kaspázou 3 sám štiepený a inaktivovaný a môže to prispievať k tomu, aby bunka zastavená v G1 fáze naopak k apoptóze smerovala (Zhang a spol., 1999). p21 sa ďalej viaže na kaspázu 8 a 10 a ďalšie transkripčné faktory stimulujúce apoptózu, napr.

Myc (Roninson, 2002). Antiapoptotická funkcia p21 sa odvíja od aktuálnej situácie v bunke a v prípade bloku bunkového cyklu vyvolaného malým poškodením DNA, kedy bunka potrebuje čas na jeho opravu, predstavuje ochrannú funkciu pred bunkovou „samovraždou".

Na rozdiel od tumor-supresorových proteínov p16 a p53, ktorých gény sú často mutované v ľudských nádoroch, p21 je mutovaný len výnimočne. V takých prípadoch je strata expresie p21 v nádoroch najčastejšie dôsledkom inaktivácie p53, ktorý je upstream efektorom p21. Podľa viacerých štúdií strata funkcie p21 koreluje s nádorovou progresiou a negatívnou prognózou napr. v prípade malobunkového pľúcneho karcinómu, kolorektálneho karcinómu, či v prípade rakoviny hlavy a krku. Paradoxne, v niektorých prípadoch bola popísaná zvýšená expresia p21 spájaná s nádorovou progresiou napr.v prípade karcinómu prostaty, vaječníkov, krčka maternice, prsníka, ap. a naznačuje jeho proonkogénnu aktivitu, v ktorej zohráva úlohu pravdepodobne jeho antiapoptotická aktivita (Roninson, 2002).

V benígnych névusoch bola detekovaná veľmi nízka alebo nulová hladina expresie p21, kdežto v metastatických vzorkách bola dokázaná vysoká úroveň expresie p21. Úloha p21 v regulácii bunkového cyklu v prípade malígneho melanómu nie je známa, a tiež prečo malígne melanocyty tolerujú vysokú hladinu p21. Carreira a spol. (2005) zistili, že v melanocytoch je transkripcia p21 aktivovaná transkripčným faktorom MITF. V našej práci (Príloha 3, str. 78) sme sa zaoberali úlohou p21 v expresii špecifických pigmentových génov a študovali sme možnú spätnoväzobnú reguláciu MITF-promótora.

2.7. mTOR signálna dráha

Signálne dráhy reprezentované kaskádovým usporiadaním proteinkináz, ako je napríklad PI3K/Akt/mTOR, sa podieľajú na regulácii bunkovej fyziológie a v ľudských nádoroch sú často deregulované. mTOR (mammalian target of rapamycin) stojí v centre signálnych dráh podieľajúcich sa na regulácii početných bunkových dejov ako je transkripcia, biogenéza ribozómov, turnover mRNA, translácia, organizácia cytoskeletu ap. (Wullschleger a Loewith, 2006).

mTOR je súčasťou dvoch heteromerických komplexov, mTORC1 a mTORC2, ktoré sa odlišujú vo svojom podjednotkovom zložení a funkciách. Lepšie preštudovaný komplex mTORC1 je zložený z mTOR, Gβ1 a proteínu raptor (regulatory associated protein of TOR) a komplex mTORC2 pozostáva z mTOR, Gβ1 a proteínu rictor (rapamycin-insensitive companion of mTOR) (Loewithe a spol., 2002).

Rapamycín, jediný selektívny inhibítor mTOR, prostredníctvom interakcie s intracelulárnym proteínom FKBP12 blokuje aktivitu komplexu mTORC1 (Sabatini a spol., 1994). Mechanizmus inhibície stále nie je úplne známy. Komplex mTORC1 je veľmi často hyperaktivovaný v mnohých typoch nádorov, a preto objavenie schopnosti rapamycínu inhibovať aktivitu mTORC1 odštartovalo množstvo klinických štúdií v liečení viacerých typov rakoviny. Obrovský potenciál rapamycínu ako protinádorového liečiva sa žiaľ nepotvrdil a zistilo sa, že pri pôsobení rapamycínu dochádza k aktivácii Akt v dôsledku aktivácie signálnej dráhy PI3K/Akt (O'Reilly a spol., 2006). Komplex mTORC2, ktorý nie je rapamycínom inhibovaný, priamo fosforyluje Akt na seríne S473 a aktivuje ju (Sarbassov a spol., 2005). V dôsledku reaktivácie Akt sa v klinických štúdiách testuje kombinácia selektívnych inhibítorov dráh mTORC1 a PI3K/Akt, ktorá by mohla odstrániť tento problém.

Vojtěchová a spol. (2008) zistili, že aktivita nereceptorovej tyrozín kinázy Src je dôležitá v regulácii dráhy mTORC1 v RSV-transformovanej bunkovej línii a inhibovali ju pomocou SU6656, farmakologického inhibítora aktivity Src. V našej práci (Príloha 4, str. 94 a Príloha 5, str. 110) sme podobne k inhibícii Src použili SU6656 a sledovali jeho účinok na dráhu Akt/mTOR v melanómovej bunkovej línii Hbl.

3. Ciele práce

Táto práca je predovšetkým zameraná na štúdium úlohy SWI/SNF komplexu v melanómových bunkách. Sleduje hlavne súvislosť s transkripčným faktorom MITF, ktorý je pre prežívanie melanómových buniek kritický. Pretože je úloha MITF tak zásadná, práca je doplnená novými poznatkami v regulácii transkripcie/aktivity MITF ďalšími mechanizmami.

Hlavné ciele:

1) Objasniť úlohu SWI/SNF chromatín-remodelujúceho komplexu v expresii MITF a prežívaní melanómových buniek v súvislosti s MITF.

Je známe, že SWI/SNF komplex je potrebný pre expresiu niektorých endogénnych cieľov MITF. Pomocou knockdownu ATPázových enzýmových podjednotiek SWI/SNF komplexu Brg1 a Brm je možné objasniť potrebu funkčného SWI/SNF komplexu v expresii samotného MITF. Medzi transkripčné ciele MITF patrí niekoľko génov s proproliferačnou a proapoptotickou funkciou. Cieľom je identifikovať gény regulované MITF, ktorých génová expresia vyžaduje funkčný SWI/SNF komplex.

2) Zistiť expresiu jednotlivých zložiek SWI/SNF komplexu v palete melanómových buniek v porovnaní s normálnymi melanocytmi.

Strata aktivity SWI/SNF komplexu je pozorovaná vo viacerých typoch nádorov. V mnohých typoch nádorov je popísaná znížená alebo úplne stratená expresia jednotlivých zložiek a znížená funkčnosť komplexov je spájaná s nádorovou progresiou, z čoho vyplýva tumor-supresorová funkcia komplexu. V poslednej dobe bola, paradoxne, popísaná aj opačná situácia, kedy expresia niektorých zložiek bola naopak zvýšená.

3) Zistiť, ktoré subkomplexy SWI/SNF sú prioritne dôležité v melanómových bunkách.

Vo všeobecnosti je v bunkách exprimovaných niekoľko subkomplexov SWI/SNF. Základné rozdelenie je podľa prítomnej ATPázovej podjednotky a môžeme ich teda rozdeliť na Brg1-obsahujúce a Brm-obsahujúce komplexy. Brm a Brg1 môžu a nemusia byť vo funkciách zastupiteľné. Cieľom je objasniť, ktorý zo subkomplexov je dôležitejší pre expresiu transkripčného faktora MITF a prežívanie melanómových buniek.

4) Určiť úlohu SWI/SNF komplexov v prežívaní melanómových buniek nezávisle na regulácii MITF a jeho cieľových génov.

Cieľom je zistiť, či existujú nejaké gény, ktoré sú zásadné pre prežívanie melanómových buniek, nie sú transkripčne regulované faktorom MITF, ale zároveň sú závislé na funkcii SWI/SNF komplexu.

5) Určiť cieľ molekulárnej génovej terapie pre melanóm.

MITF a SWI/SNF komplex se zdajú byť vhodnými cieľmi génovej terapie, ich inaktivácia však musí byť cielená výhradne do nádorových buniek.

Doplnkové ciele:

6) Určiť úlohu p21 v transkripcii MITF.

Presné definovanie transaktivačnej funkcie MITF môže prispieť k cielenej inhibícii jeho aktivity, na ktorej je prežívanie melanómových buniek závislé. Jedným z transkripčných cieľov MITF je inhibítor bunkového cyklu p21. Ďalšou úlohou je objasniť spätnoväzobnú reguláciu MITF-promótora proteínom p21. Tento mechanizmus môže pomáhať udržiavať hladinu MITF, a tým prispievať k expresii antiapoptotických a antiproliferačných génov v melanómových bunkách (viď Úvod a literárny prehľad).

7) Objasniť úlohu PCAF a hGCN5 v regulácii MITF na melanocytovo-závislom melastatinovom promótore.

K epigenetickým mechanizmom regulujúcim génovú expresiu patria okrem chromatín-remodelujúcich komplexov aj komplexy s HAT aktivitou. Cieľom je zistiť, či histón-acetyltransferázy PCAF a hGCN5 pôsobia ako kofaktory MITF pre jeho transkripčnú aktivitu na cieľových promótorch.

8) Určiť účinok SU6656 na aktivitu mTOR.

Medzi hyperaktivované signálne dráhy v malígnych melanómoch patrí dráha PI3K-Akt, ktorej efektorom je mTOR. Cieľom je vyšetriť možnosť inhibície tejto dráhy prostredníctvom špecifického inhibítora Src-kinázovej aktivity, SU6656.

4. Použité metódy

Podrobnosti k jednotlivým metódam sú uvedené v príslušných prílohách.

Kultivácia buniek

viz. Príloha 1 (str. 51), 2 (str. 57), 3 (str. 78)

Plazmidy, expresné a promótorové konštrukty

viz. Príloha 1 (str. 51), 2 (str. 57), 3 (str. 78), 8 (str. 144)

Transfekcie a reportérové štúdie

viz. Príloha 1 (str. 51), 2 (str. 57), 8 (str. 144)

shRNA knock-down a lentivírusová infekcia

viz. Príloha 1 (str. 51), 2 (str. 57), 3 (str. 78)

RT-PCR a "real time" PCR

viz. Príloha 1 (str. 51), 2 (str. 57), 3 (str. 78)

Western blotting

viz. Príloha 1 (str. 51), 2 (str. 57), 3 (str. 78)

Imunoprecipitácia

viz. Príloha 4 (str. 94), 8 (str. 144)

Imunohistochémia

viz. Príloha 2 (str. 57)

Chromatín-imunoprecipitácia

viz. Príloha 1 (str. 51), 3 (str. 78)

"Colony formation assay", "viability assay" a proliferačné krivky

viz. Príloha 1 (str. 51), 2 (str. 57), 4 (str. 94)

"Soft agar colony assay"

viz. Príloha 4 (str. 94)

Prietoková cytometria

viz. Príloha 2 (str. 57)

"Microarray" analýza

viz. Príloha 2 (str. 57)

Príprava "GST-fusion" proteínov a "Pull-down" analýza

viz. Príloha 8 (str. 144)

5. Výsledky

Výsledky sú podrobne spracované v jednotlivých prílohách. V nasledujúcom texte budú stručne zhrnuté len najdôležitejšie výsledky.

5.1. Expresia jednotlivých podjednotiek SWI/SNF komplexu v melanómových bunkách

Analýzou jadrových extraktov 8 melanómových línií a 3 nenádorových melanocytových línií sme pomocou Western blotu určili hladiny 10 zložiek SWI/SNF komplexov (Fig. 1, Príloha 1, str. 52). Zistili sme vysokú expresiu väčšiny podjednotiek v melanómových bunkách v porovnaní s normálnymi melanocytmi. Z analyzovaných línií len línia SK-MEL-5 postrádala expresiu enzýmovej podjednotky Brg1. Brm bol exprimovaný vo všetkých testovaných líniách. Vo všeobecnosti majú melanómové bunky zachovanú alebo dokonca zvýšenú hladinu SWI/SNF proteínov.

5.2. Brm a Brg1 aktivujú MITF-promótor a viažu sa na MITF-promótor *in vivo*

Medzi transkripčné faktory aktivujúce promótor génu pre MITF patria transkripčné faktory Pax3, Sox10, CREB a LEF-1/TCF (Vachtenheim a Borovanský, 2010). Brg1 interaguje s β-katenínom pri aktivácii TCF transkripčných faktorov (Barker a spol., 2001) a naznačuje potrebu Brg1 v transkripčnej regulácii MITF-promótora. V promótor-reportérovom systéme sme sledovali účinok exogénnej hladiny Brg1, Brm a ich dominantne negatívnych mutantov (bez ATPázovej aktivity) na aktivitu MITF-promótora. Zvýšenie hladiny proteínu Brg1 alebo Brm prostredníctvom expresných vektorov malo za následok zvýšenie promótorovej aktivity. V prípade dominantne negatívnych mutantov sme pozorovali mierne inhibičný účinok (Fig. 2A, Príloha 1, str. 53). Úloha Brg1 a Brm v iniciácii transkripcie MITF bola potvrdená pomocou chromatín-imunoprecipitácie (Fig. 2B, Príloha 1, str. 53). Zistili sme, že v bunkových líniách 501mel a Hbl, v ktorých sú oba proteíny Brm i Brg1 veľmi dobre exprimované, sa na promótorovú oblasť génu pre MITF viaže proteín Brg1. V bunkách SK-MEL-5, ktoré nemajú exprimovaný Brg1, sme detekovali na proximálnej oblasti MITF-promótora naviazaný proteín Brm. Tieto výsledky potvrdzujú asociáciu Brm a Brg1 s iniciačným komplexom na MITF-promótore a naznačujú, že primárne rekrutovaným proteínom v Brm/Brg1-pozitívnych melanómových bunkách je proteín Brg1.

5.3. Brg1 dramaticky znižuje expresiu MITF v melanómových bunkách 501mel

V logickom slede s predchádzajúcimi výsledkami sme študovali úlohu enzýmových podjednotiek Brm a Brg1 v expresii transkripčného faktora MITF. Pomocou Western blotu sme analyzovali hladinu expresie proteínu MITF po umlčaní expresie Brg1, Brm alebo oboch prostredníctvom shRNA a zistili sme obrovský pokles expresie MITF v Brg1-umlčaných bunkách 501mel (Fig. 3A, Príloha 1, str. 54). Umlčanie hladiny Brm malo v tomto prípade len slabý účinok. Na rozdiel od 501mel, v Brg1-neexprimujúcej línii SK-MEL-5 bol pokles hladiny Brm postačujúci na potlačenie expresie MITF (Fig. 3C, Príloha 1, str. 54). Účinok knockdownu Brg1 a Brm sme overovali na úrovni mRNA a pomocou real time PCR sme analyzovali v shBrg1- a shBrm-transfektovaných bunkách 501mel hladinu mRNA MITF a jeho troch transkripčných cieľov (melastatinu, CDK2 a tyrozinázy) (Fig. 3D, Príloha 1, str. 54). V oboch prípadoch, knockdown Brg1 i Brm mal za následok pokles analyzovaných hladín mRNA v porovnaní s kontrolou, ale v prípade Brg1 bol tento efekt výraznejší. Súhrnne tieto výsledky ukazujú, že SWI/SNF komplex je skutočne kritický faktor pre expresiu MITF a úlohu primárneho kofaktora MITF zohrávajú pravdepodobne SWI/SNF komplexy asociované s Brg1. Preto sme sa v ďalšej práci dôkladnejšie venovali inaktivácii práve tejto enzýmovej podjednotky.

5.4. Knockdown Brg1 a Brm inhibuje proliferáciu a znižuje viabilitu

Keďže vysoká hladina MITF je spájaná s proliferáciou a prežívaním melanómových buniek, analyzovali sme rastové krivky Brg1-, Brm- aj Brg1/Brm-umlčaných buniek (Fig. 4A, Príloha 1, str. 55). Pre efektívnejšie umlčanie expresie Brg1, Brm či oboch proteínov Brg1 i Brm súčasne (vďaka vysokej homológii Brg1 a Brm bolo možné použiť spoločnú sekvenciu shBrg1/Brm), sme používali lentivírusový prenos shRNA zabezpečujúci stabilnejšie umlčanie expresie v porovnaní s plazmidovými expresnými vektormi pre shRNA. Najvýraznejší inhibičný účinok na proliferáciu melanómových buniek 501mel mal knockdown Brg1. Proliferačný blok bol po 6 dňoch prekonaný a bunky začali znova proliferovať. Predpokladali sme, že obnovená proliferácia po niekoľkých dňoch bola v dôsledku schopnosti proteínu Brm kompenzovať funkciu Brg1. Pomocou Western blotu sme potvrdili, že v Brg1-umlčaných bunkách 501mel sa po niekoľkých dňoch skutočne re-exprimoval MITF (Fig. 4B, Príloha 1, str. 55). Predpoklad kompenzačnej funkcie Brm sme overili pomocou experimentu, v ktorom sme najskôr umlčali expresiu Brm a následne sme bunky reinfikovali shBrg1-lentivírusom. Narozdiel od buniek reinfikovaných kontrolným vírusom, shBrg1-reinfikované bunky neboli schopné proliferovať (Fig. 4C v pravo, Príloha 1, str. 55). Tieto výsledky ukazujú, že napriek

tomu, že Brg1 je primárne dôležitejší pre proliferáciu melanómových buniek, Brm je schopný kompenzovať jeho funkciu. Avšaj pre proliferáciu melanómových buniek je potrebná expresia aspoň jednej ATPázovej podjednotky SWI/SNF komplexu. Potrebu aspoň jednej ATPázy SWI/SNF komplexu v melanómových bunkách potvrdzujú výsledky z testu viability (Fig. 2, Príloha 2, str. 59), kde najefektívnejší dopad na zníženie viability malo umlčanie expresie oboch proteínov Brm a Brg1 súčasne.

5.5. Knockdown Brg1 indukuje apoptózu v 501mel

Je známé, že umlčanie expresie MITF v bunkách 501mel spôsobuje zastavenie bunkového cyklu (Carreira a spol., 2006). Analýzou bunkového cyklu shMITF- a shBrg1-transfektovaných buniek 501mel (po 3 dňovej selekcii v puromycíne) pomocou flow cytometrie sme zistili nárast bunkovej populácie v sub-G fáze indikujúci apoptózu (Fig. 1A, Príloha 2, str. 59). Výsledky sme potvrdili pomocou "TUNEL" analýzy. Ďalej sme zistili veľmi slabú aktiváciu kaspázy 3 a 7 a nedetekovali sme štiepenie proteínu PARP, z čoho vyplýva, že apoptóza v sh-Brg1- a sh-MITF-transfektovaných bunkách 501mel bola aktivovaná cestou nezávislou na kaspázach.

5.6. Microarray analýza génovej expresie v Brg1-knockdownovaných bunkách 501mel

Pomocou celogenómového skríningu (cDNA microarray) sme zisťovali transkripčnú odpoveď po akútnom zablokovaní Brg1 v 501mel bunkách. Rozhodli sme sa pre knockdown Brg1, pretože z predchádzajúcich výsledkov sme určili Brg1 ako primárnu ATPázu SWI/SNF komplexu v regulácii expresie MITF (viď vyššie). Následkom deplécie Brg1 bol MITF downregulovaný približne 5-krát. Ďalej sme v analýze zistili viac ako 2-násobný pokles v hladine mRNA 381 génov (reprezentovaných 485 próbami) ($P<0,005$) (tabuľka S1, Príloha 2, str. 69) a viac ako 2-násobný nárast v hladine mRNA 210 génov (reprezentovaných 273 próbami) ($P<0,005$) (tabuľka S3, Príloha 2, str. 75). Reprimované gény, ktoré patria medzi transkripčné ciele MITF sú zhrnuté v tabuľke S2 (Príloha 2, str. 74). Gény aktivované alebo reprimované viac ako 8-krát (67 génov, ($P<0,005$)) sú zobrazené na obrázku Fig. 3A (Príloha 2, str. 60). Medzi výrazne reprimovanými génmi je gén pre IGF1 (insulin-like growth factor 1) a osteopontin (OPN), faktory známe svojou antiapoptotickou, proproliferačnou a proinvazívnou aktivitou v melanómoch (Zhou a spol., 2005; Hilmi a spol., 2008), a tiež TGFβ2 (transforming growth factor β2), ktorého zvýšená expresia je detekovaná vo viacerých typoch nádorov vrátane melanómov (Javelaud a spol., 2008). Okrem týchto troch sme zistili represiu ďalších faktorov (survivin, E2F1, p15), ktoré majú úlohu v prežívaní

melanómových buniek a nie sú transkripčne regulované MITF. Výsledky z microarray analýzy potvrdzujú dôležitú úlohu Brg1 v zachovaní transkripcie regulovanej prostredníctvom MITF a odhaľujú úlohu Brg1 v regulácii ďalších faktorov s funkciou v prežívaní melanómových buniek pôsobiacich mimo os MITF.

Zníženú expresiu mRNA MITF-nezávislých faktorov OPN, TGFβ2, survivín a IGF1 v shBrg1-transfektovaných bunkách 501mel sme overili na úrovni proteínovej expresie pomocou Western blotu, a ďalej sme potvrdili nezávislosť expresie týchto faktorov na expresii MITF (Fig. 4, Príloha 2, str. 61). Podrobnejšie spracovanie je v Prílohe 2 (str. 57). Reintrodukcia OPN, CDK2 a IGF1 do shBrg1-transfektovaných buniek čiastočne obnovila proliferáciu nádorových buniek (Fig. 6, Príloha 2, str. 63). Súhrnne môžeme povedať, že blokovanie funkcie SWI/SNF komplexov v melanómových bunkách vedie k inhibícii rastu v dôsledku komplexného poklesu hladín „prežívacích" faktorov.

5.7. Brg1 a Brm sú exprimované v melanómoch a névusoch

Imunohistochemickou analýzou (Fig. 5, Príloha 2, str. 62) sme zistili, že Brm je veľmi dobre exprimovaný v névusoch aj vo vzorkách primárnych melanómov a neboli detekované žiadne Brm-negatívne bunky. Brg1 expresia ukázala heterogénnosť vo vzorkách melanómov v porovnaní s benígnymi névusmi. V súlade s predchádzajúcimi výsledkami (Carreira a spol., 2006) sa nám potvrdila heterogénna expresia MITF v névusoch aj v melanómoch.

5.8. p21 je exprimovaný v melanómových bunkách, aktivuje MITF promótor a viaže sa na promótor MITF *in vivo*

Zistili sme, že p21 je dobre exprimovaný v normálnych ľudských melanocytoch a aj v melanómových bunkách (Fig. 2A, Príloha 3, str. 81). Okrem toho sme potvrdili koreláciu medzi hladinou proteínu p21 (analyzovaná pomocou Western blotu) a hladinou mRNA MITF (analyzovaná pomocou real time PCR) v 7 z 10 melanómových línií (Fig. 2B, Príloha 3, str. 81). Keďže p21 funkčne pôsobí v bunke aj nezávisle na jeho funkcii inhibítora bunkového cyklu, zaujímal nás regulačný účinok p21 v melanocytovo-špecifickej transkripcii. V promótor-reportérových systémoch sme zisťovali účinok koexpresie p21 na aktivitu promótora MITF a troch cieľových promótorov MITF (tyrozinázy, melastatinu a TRP1) v 4 bunkových líniách (MeWo, SK-MEL-5, SK-MEL-28 a B16-F10). Ako vyplýva z obrázku Fig. 1A (Príloha 3, str. 80), exogénny p21 stimuluje aktivitu promótora MITF a promótora tyrozinázy a melastatinu. Stimulácia cieľových promótorov MITF je pravdepodobne v dôsledku dočasného zvýšenia hladiny MITF v transfektovaných bunkách. Pomocou

chromatín-imunoprecipitácie sme potvrdili, že p21 sa viaže na promótorovú oblasť génu pre MITF (Fig. 5B, Príloha 3. str. 86). V tomto experimente boli použité melanómové bunky Hbl s vysokou expresiou p21 a ako negatívna kontrola melanómové bunky 501mel s nulovou expresiou p21. Výsledky potvrdzujú asociáciu p21 s iniciačným komplexom na promótore pre MITF. V Prílohe 3 (str. 78) je táto problematika podrobnejšie spracovaná vrátane kooperácie p21 s proteínom CREB pri aktivácii MITF-promótora.. Z uvedených zistení vyplýva ďalšia funkcia p21 ako kofaktora v transkripcii MITF nezávisle na inhibícii aktivity cyklín-CDK komplexov.

5.9. PCAF a hGCN5 interagujú s MITF a inhibujú aktivitu melastatinového promótora

Medzi známe koaktivátory MITF patria histón-acetyltransferázy CBP/P300 (Sato a spol, 1997; Price a spol., 1998). V našej práci sme sa zamerali na ďalšie dva HAT enzýmy, PCAF a hGCN5, ktoré by potenciálne tiež mohli plniť funkciu koaktivátorov MITF. Tieto proteíny sú vysoko homológne a sú súčasťou multienzýmových komplexov acetylujúcich históny a nehistónové proteíny (napr. transkripčné faktory), a tým môžu posttranslačne ovplyvňovať ich aktivitu (Nagy a Tora, 2007). V našej práci sme pomocou metódy pull-down zistili, že PCAF a hGCN5 interagujú s MITF *in vitro*, a konkrétne je pre túto interakciu nevyhnutná bHLH oblasť proteínu MITF. Pre tento účel sme pripravili pomocou *in vitro* translácie (IVT) 11 rôznych delečných mutantov MITF značených ^{35}S-metionínom a rekombinantné proteíny GST-PCAF, GST-hGCN5 a ako pozitívnu kontrolu GST-Rb (pRb interaguje s MITF (Carreira a spol., 2005)). (Výsledky pull-downu sú podrobnejšie spracované v Prílohe 8, str. 146).

Pomocou imunoprecipitácie sme potvrdili interakciu *in vivo* (Fig. 1D, Príloha 8, str. 155). V promótor-reportérových štúdiách sme zistili, že ektopický PCAF a hGCN5 inhibujú aktivitu melastatinového promótora (MITF-cieľového promótora) nezávisle na ich histón-acetyltransferázovej aktivite (Fig. 2, Príloha 8, str. 156). Umlčanie endogénnej hladiny PCAF pomocou siRNA duplexov v melanómových bunkách 501mel malo za následok nárast aktivity melastatinového promótora (Fig. 5, Príloha 8, str. 159), z čoho možno usúdiť, že endogénna hladina PCAF má inhibičný účinok na MITF-cieľový melastatinový promótor. Výsledky sú podrobnejšie spracované v Prílohe 8 (str. 144) a sú pripravované pre publikáciu do odbornej tlače. K tejto časti práce ešte musí byť dokončená chromatín-imunoprecipitácia, ktorá by potvrdila, že PCAF a hGCN5 sú rekrutované k MITF-cieľovým promótorom a podporila by hypotézu o represorovej funkcii PCAF a hGCN5 na aktivitu transkripčného faktora MITF.

5.10. Inhibícia mTORC1 pomocou selektívneho Src-kinázového inhibítora SU6656 v melanómových bunkách

Analýzou niekoľkých melanómových línií sme zistili, že v porovnaní s normálnymi melanocytmi majú hyperaktivovanú mTOR-signálnu cestu, podobne ako je to v mnohých ľudských nádoroch. Upstream regulátor mTOR, serín/treonín kináza Akt interaguje s podjednotkami SWI/SNF komplexu, INI1 a BAF155 a v kinázovej reakci *in vitro* tieto proteíny fosforyluje (Foster a spol., 2006). V tejto časti sme nesledovali priamy súvis so SWI/SNF komplexom, ale pokúsili sme sa zablokovať túto signálnu cestu pomocou selektívnych inhibítorov.

Pomocou analýzy zmien fosforylácie kľúčových proteínov dráhy mTORC1 použitím Western blotu sme potvrdili pozitívny účinok rapamycínu na umlčanie dráhy mTORC1 v melanómových bunkách Hbl. Zistili sme pokles vo fosforylácií mTOR (Ser2448), efektorového proteínu S6 kinázy (S6K1) a jej cieľov, ribozomálneho proteínu S6 (Ser235/236) a proteínu rictor (Thr1135), ktorý je súčasťou komplexu mTORC2 (Fig. 1A, Príloha 4, str. 109). Podľa očakávaní viedlo ovplyvnenie melanómových buniek rapamycínom k zvýšeniu aktivity dráhy Akt reprezentovanej zvýšenou fosforyláciou Akt na Ser473, a tiež jej substrátov tuberínu a MAPK (Fig. 1B, Príloha 4, str. 109).

Ovplyvnenie buniek Hbl špecifickým inhibítorom Src-kináz SU6656 malo na dráhu mTORC1 podobný účinok (Fig. 2A, Príloha 4, str. 109). Na rozdiel od rapamycínu, SU6656 nevyvolal spätnou väzbou hyperaktiváciu Akt, ale práve naopak, pozorovali sme pokles fosforylácie Akt na Ser473 v porovnaní s kontrolnými bunkami a výsledky naznačujú, že Akt by mohol byť substrátom Src. Pomocou imunoprecipitácie sme zistili prítomnosť Src v imunoprecipitáte proteínu raptor, podjednotky komplexu mTORC1 a dokázali sme tak fyzickú interakciu Src s mTORC1 komplexom. Asociáciu Src s komplexom mTORC2 sa nám potvrdiť nepodarilo (Fig. 3, Príloha 4, str. 109). Prítomnosť Src v komplexe mTORC1 je úplne novým zistením a v ďalšej práci je nutné objasniť funkčný dopad prítomnosti Src na aktivitu komplexu mTORC1.

6. Diskusia

V predkladanej práci sa predovšetkým zaoberám úlohou SWI/SNF chromatín-remodelujúceho komlexu v melanómových bunkových líniách so zameraním na jeho enzymatické podjednotky Brm a Brg1 a jej súvislosťou s "master" regulátorom normálnych i nádorových melanocytov, transkripčným faktorom MITF. Úloha SWI/SNF komplexu zasahuje do mnohých bunkových procesov, a hoci je považovaný za tumorový supresor, v nádorovej transformácii môže vykazovať protichodné účinky, tkanivovo- prípadne bunkovo-špecifické.

V súčasnosti stále nie sú celkom jasné mechanizmy regulujúce expresiu a transkripčnú aktivitu MITF počas melanocytového vývoja, v dospelých melanocytoch, či v prípade malígneho melanómu. Popísali sme kritickú úloha SWI/SNF komplexu v regulácii expresie MITF (Príloha 1, str. 54). Na tieto výsledky nadväzuje a význam SWI/SNF umocňuje ďalšia práca (Príloha 2, str. 60), kde je popísaná aj jeho funkcia nezávisle na MITF. Jeho MITF-nezávislá funkcia by mohla byť prínosnou v prístupe k terapii melanómu a môže preklenúť problém vznikajúci pri potenciálnej génovej terapii zameranej na MITF ako kľúčový líniovo-špecifický onkogén.

Hoci v niekoľkých nádorových bunkových líniách bola pozorovaná strata expresie oboch enzýmových podjednotiek Brm a Brg1 (DeCristofaro a spol., 2001), v melanómových líniách sú oba veľmi dobre exprimované. Charakterizovali sme 9 melanómových línií a našli sme len jednu, SK-MEL-5, ktorá postrádala expresiu Brg1. Brm bol exprimovaný vo všetkých testovaných líniách (Fig. 1, Príloha 1, str. 52). Keenen a spol. (2010) vo svojej práci charakterizovali 8 melanómových línií a podobne ako naša skupina, popísali Brg1-negatívnu líniu SK-MEL-5 a ďalšie dve s veľmi nízkou expresiou Brm (PMWK a YUMAC). V našej práci sme imunochemickou analýzou zistili, že oba proteíny sú veľmi dobre exprimované vo vzorkách névusov aj primárnych melanómov. Expresia Brg1 je viac heterogénna v primárnych melanómoch v porovnaní s névusmi, avšak neboli detekované žiadne Brm-negatívne bunky v névusoch ani primárnych melanómoch (Fig. 5, Príloha 2, str. 62). Becker a spol. (2009) síce zistili stratu expresie Brg1 v 12 z 38 vzoriek melanómov (32%) a stratu expresie Brm v 10 z 50 vzoriek melanómov (20%), avšak Lin a spol. (2010), ktorých práca vychádzala z ďaleko širšej palety vzoriek (185), publikovali signifikantný nárast expresie Brg1 v prípade primárnych i metastatických melanómov v porovnaní s dysplastickými névusmi. Dôkladnešia imunochemická analýza expresie Brm by mohla

odhaliť podobné výsledky. Je možné, že v neskorších štádiách metastatického melanómu bunky majú downregulovaný Brg1 a kritickou pre funkčný SWI/SNF komplex zostáva expresia Brm. V každom prípade si melanómové bunky zachovávajú expresiu aspoň jednej enzymatickej podjednotky.

Vo všeobecnosti sa pozornosť čoraz viac sústreďuje na chromatínové modifikácie ovplyvňujúce prístupnosť promótorovej oblasti cieľového génu. Ozsolak a spol. (2007) pomocou metódy vysoko rozlišujúcej chromatínovej analýzy zistili, že transkripčný faktor MITF sa prednostne viaže na oblasti bez nukleozómov. Tieto výsledky sú v súlade s potrebou remodelácie chromatínu promótorových oblastí cieľových génov MITF prostredníctvom SWI/SNF, ktorý zohráva úlohu dôležitého kofaktoru MITF.

Naše výsledky jasne potvrdzujú, že expresia transkripčného faktora MITF je absolútne závislá na funkcii chromatín-remodelujúcich komplexov SWI/SNF (Príloha 1, str. 51) a poukazujú na úlohu epigenetickej regulácie v tumorogenéze malígneho melanómu. Primárne dôležitou v expresii MITF sa javí enzymatická podjednotka Brg1, ktorá sa prednostne viaže na proximálnu oblasť MITF-promótora v Brm/Brg1-exprimujúcich melanómových líniách. Toto potvrdzujú aj výsledky knockdown experimentov, keďže sme v bunkách s umlčanou expresiou Brg1 detekovali výraznejší pokles hladín mRNA MITF a jeho 3 transkripčných cieľov (cdk2, melastatinu a tyrozinázy) v porovnaní s Brm-knockdownovanými bunkami. Nepriama úloha Brg1 v expresii MITF čiastočne vyplýva už zo súvislosti s β-katenínovou dráhou, ktorá je v melanómoch často deregulovaná (Widlund a spol., 2002; Sinnberg a spol., 2011; Delmas a spol., 2007). Brg1 interaguje s β-katenínom a stimuluje transkripčnú aktivitu efektorov β-katenínovej cesty. Jedným z efektorov β-katenínovej dráhy je transkripčný faktor LEF1/TCF, známy koaktivátor expresie MITF (Barker a spol., 2001).

Už predtým bolo zistené, že funkčný SWI/SNF komplex ovplyvňuje niektoré špecifické melanocytové gény. Brg1 bol spolu s MITF detekovaný na promótoroch trp1 a tyrozinázy a pomocou imunoprecipitácie a imunofluorescencie bola potvrdená ich fyzická interakcia a spoločná lokalizácia v jadre (de la Serna a spol., 2006). Keenen a spol. (2010) zistili, že Brg1 sa viaže na promótory závislé na transkripčnej aktivite MITF, napr. promótor pre ML-IAP, tyrozinázu, trp1, trp2, rab27A a p21. V tejto práci autori ďalej zistili, že po umlčaní expresie MITF, Brg1 nie je schopný viazať sa na MITF-závislé promótory. MITF pri aktivácii cieľových promótorov potrebuje rekrutované funkčné Brg1-obsahujúce SWI/SNF komplexy. Z uvedených poznatkov a našich zistení môžeme považovať Brg1

za enzýmovú podjednotku primárne dôležitú v expresii MITF a niektorých jeho cieľových génov.

Hoci Brm a Brg1 sú vo svojich funkciách častokrát nezastupiteľné, napr. počas embryonálneho vývoja, bolo popísaných niekoľko prípadov, kedy sa dokážu vo svojich funkciách navzájom kompenzovať. Napr. v prípade Brm/Brg1-deficientných bunkových línií SW13 a A427, ktoré sú odolné voči pRb-sprostredkovanému zastaveniu bunkového cyklu. Re-introdukcia Brm alebo Brg1 do týchto buniek obnovila citlivosť pRb-signálnej dráhy s následným zastavením bunkového cyklu (Strobeck a spol., 2002). Xu a spol. (2002) zistili, že Brg1, ale nie Brm, je potrebný pre indukciu p21 v MCF7 bunkách. Po stabilnom knockdowne Brg1 v tejto bunkovej línii bola jeho funkcia kompenzovaná proteínom Brm. Kompenzačnú úlohu Brm sme pozorovali aj my v melanómovej línii 501mel po stabilnom knockdowne Brg1. Po počiatočnej inhibícii proliferácie a poklese expresie MITF v dôsledku umlčanej expresie Brg1 začali bunky po niekoľkých dňoch proliferovať a expresia MITF sa obnovila (Fig. 4B, Príloha 1, str. 55). Kompenzačná úloha Brm je zrejmá aj v prípade melanómovej línie SK-MEL-5, v ktorej nie je exprimovaný Brg1 (Fig. 1, Príloha 1, str. 52). Strata expresie MITF a inhibícia proliferácie po umlčaní expresie Brm v SK-MEL-5 jasne dokazuje, že melanómové bunky potrebujú pre svoju viabilitu exprimovanú aspoň jednu ATPázovú podjednotku SWI/SNF komplexu.

Pomocou cDNA microarray analýzy Brg1-umlčaných buniek 501mel sme zistili, že knockdown Brg1 má za následok pokles expresie nielen proproliferačných faktorov závislých na MITF (cdk2, livin), ale tiež ďalších proproliferačných a pro-invazívnych faktorov, ako je IGF1, osteopontin, TGFβ2, survivin a SOX10, ktoré sú nezávislé na MITF (Príloha 2, str. 51). Z výsledkov je zrejmé, že knockdown Brg1 inhibuje proliferáciu melanómových buniek aj nezávisle na dráhe MITF. V súlade s týmto poznatkom je aj zistenie, že re-expresia plazmidového konštruktu hyperaktívneho mutanta MITF-Vp16 nedokázala obnoviť bunkový rast (Fig. 6B, Príloha 2, str. 63), pretože expresia proproliferačných downstream efektorov MITF potrebuje funkčný SWI/SNF komplex (Keenen a spol., 2010) a v prežívaní hrajú úlohu ďalšie, na MITF-nezávislé faktory. Príkladom dôležitej funkcie Brg1 v progresii melanómu mimo os MITF je napríklad jeho schopnosť modulovať expresiu niektorých adhezívnych proteínov a skupiny enzýmov podieľajúcich sa na remodelácii extracelulárnej matrix. Brg1 aktivuje expresiu metaloproteinázy (MMP) 2, ktorá prispieva k zvýšenej invazivite melanómových buniek. Chromatín-imunoprecipitáciou bola dokázaná lokalizácia Brg1 na promótore MMP2, ktorá potvrdzuje priamu aktiváciu expresie tohoto génu asociovaného s metastázami (Saladi a spol., 2010b). Naidu a spol. (2009) zisitili, že Brg1 a p300 tlmia

aktivitu p53 v nádoroch exprimujúcich divoký typ proteínu p53. Umlčanie expresie Brg1 (ale nie Brm) aktivuje endogénny wt p53 a senescenciu. V melanómových bunkách podobná súvislosť nebola skúmaná, ale treba podotknúť, že p53 býva v melanómoch mutovaný len zriedka (Gray-Schopfer a spol., 2007), a preto by mohol Brg1 podobným spôsobom zvyšovať prežívanie melanómových buniek bez mutovaného p53. Môžeme teda zhrnúť, že nami pozorovaná úloha Brg1 v melanómovej progresii vyplývajúca z upregulácie množiny faktorov spájaných s prežívaním nádorových buniek je v súlade s jeho proonkogénnymi účinkami pozorovanými inými autormi, a hoci významnú úlohu predstavuje manipulácia hladiny MITF s následnou reguláciou jeho transkripčnej aktivity, mnoho účinkov je realizovaných mimo dráhu MITF.

Kang a spol. (2004) zistili že Brg1-obsahujúce SWI/SNF komplexy regulujú bunkový cyklus prostredníctvom regulácie p21 nezávisle na interakcii Brg1 s pRB. Brg1 prostredníctvom upregulácie p21 udržuje pRb v hypofosforylovanom a teda aktívnom stave. K podobným výsledkom dospeli Hendricks a spol. (2004), ktorí reintrodukciou Brg1 do bunkovej línie rakoviny prsníka s definovanou mutáciou Brg1 génu indukovali zastavenie bunkového cyklu a zistili, že sa Brg1 priamo viaže na promótor p21 nezávisle na p53. Je dôležité poznamenať, že zastavenie bunkového cyklu prostredníctvom Brg1-stimulovanej expresie p21 bolo pozorované v nádorových líniách so zníženou expresiou/aktivitou Brg1. Pre niektoré línie plní Brg1 funkciu nádorového supresora a stráta jeho expresie/aktivity podporuje nádorovú progresiu. Situácia môže byť odlišná v nádoroch s proonkogénnou funkciou SWI/SNF komplexov, ako je napr. prípad malígneho melanómu. Okrem zvýšenej expresie zložiek SWI/SNF komplexu bola v melanómových líniách pozorovaná aj zvýšená expresia p21 (Carreira a spol., 2005; naše výsledky Príloha 3, str. 78), ktorá by mohla byť spájaná s proonkogénnou aktivitou (viď Úvod a literárny prehľad). Stimulácia expresie p21 prostredníctvom zvýšenej expresie Brg1 by teda mohla prispievať k zvýšenej rezistencii malígneho melanómu voči apoptóze. Táto hypotéza vyžadujúca podrobnejšie skúmanie by mohla vytvoriť komplexný rámec s našimi výsledkami (proproliferačný účinok spätnoväzobnej regulácie promótora MITF a p21 (Príloha 3, str. 78) a proproliferačný účinok Brg1 MITF-závislou dráhou (Príloha 1, str. 51)).

Ako už bolo niekoľkokrát uvedené, MITF je kľúčovým transkripčným faktorom a líniovo-špecifickým onkogénom melanocytov (Levy a spol, 2006). Protichodné úlohy jeho transkripčných cieľov v bunkových procesoch si prirodzene vyžadujú veľmi citlivú reguláciu jeho expresie i jeho aktivity. Súčasné poznatky vedú k tzv. reostatovému modelu expresie MITF, v ktorom hladina MITF predstavuje rozdielne biologické fenotypy. Na jednom konci

je terminálna diferenciácia s vysokou hladinou MITF a na druhom konci apoptóza a bunková smrť s nulovou expresiou MITF. Hladiny medzi predstavujú vratné, dynamické deje. Nie je teda prekvapivé, že vo vzorkách melanómov je expresia MITF značne heterogénna. Melanómové bunky s nízkou hladinou MITF sa vyznačujú vysokou invazivitou a nízkou proliferáciou, kdežto rýchlo proliferujúce bunky majú značne vysoké hladiny MITF (Carreira a spol., 2006). Hladina MITF v melanómoch a jeho aktivita je pravdepodobne závislá na mikroprostredí a dynamicky ovplyvňuje charakter melanómových buniek. Expresia MITF je značne heterogénna aj v melanómových líniách, ktoré sa líšia svojou citlivosťou na umlčanie expresie MITF (Kido a spol., 2009). Preto, v dôsledku využívania širokej palety melanómových línií v experimentálnej práci, nie je funkčný význam endogénnej hladiny MITF v melanómových bunkách jednoznačne interpretovaný. Naše zistenie, že knockdown MITF v línii 501mel indukuje apoptózu (Fig 1A, Príloha 2, str. 59), je v rozpore s inými výsledkami (Giuliano a spol., 2010; Ohanna a spol., 2011), kde inhibícia expresie MITF indukovala senescenciu. Odlišné výsledky možno vysvetliť rozdielom v použitej metodike, konkrétne prevedením RNA-interferencie. Nami prevedená stabilná transfekcia plazmidov exprimujúcich veľmi účinnú sh-RNA proti MITF je v porovnaní s dočasnou transfekciou RNA duplexov proti MITF (Giuliano a spol., 2010) výrazne efektívnejšia v umlčaní expresie MITF. V súlade s reostatovým modelom, aj veľmi malá zostatková hladina MITF môže byť pre melanómové bunky postačujúca a viesť k senescencii, zatiaľ čo nulová hladina môže indukovať apoptózu. Postačujúca veľmi nízka hladina MITF k regulácii proliferácie bola pozorovaná napr. v melanómovej línii A375 (Kido a spol., 2009). Melanómové línie IgR3 a RPMI1795 pochádzajúce údajne z desmoplastického melanómu majú síce nulovú hladinu MITF, ale sú silno dediferencované, bez expresie melanocytovo-špecifických génov a pripomínajú viac nemelanocytové línie. Reintrodukcia MITF do takýchto buniek neobnovila melanocytový fenotyp (Vachtenheim a spol., 2001), a je otázne, či ich možno ešte považovať za melanómové.

V dôsledku heterogénnej expresie MITF v nádoroch a taktiež kvôli rozdielnej senzitivite jednotlivých melanómových línií, by bolo preto pravdepodobne účinnejšie použiť v génovej terapii cielené umlčanie aktivity proonkogénne pôsobiaceho SWI/SNF komplexu, obzvlášť subkomplexu obsahujúceho Brg1. Ako sme zistili, vyradenie jeho funkcie taktiež vedie k silnej apoptóze s následným neprežívaním melanómových buniek (Fig 1A, Príloha 2, str. 59) a hladiny Brg1 i Brm sú vo vzorkách primárnych melanómov vysoké. Problém by mohol predstavovať technický dizajn terapie, konkrétne zacielenie vhodných terapeutickych vektorov do nádoru, pretože komponenty SWI/SNF komplexu sú exprimované vo všetkých

bunkách ľudského organizmu. Ďalej by prichádzala do úvahy kombinácia s ďalšími prístupmi, napr. použitím selektívnych inhibítorov signálnej dráhy MEK-ERK alebo dráhy PI3K-Akt-mTOR, ktoré sú často hyperaktivované v melanómoch. (Pilotné výsledky s použitím selektívneho inhibítora Src v melanómových bunkách sú diskutované nižšie).

Rozpoznanie mechanizmov transkripčnej aktivácie MITF a identifikácia ďalších cieľových génov súvisiacich s kontrolou proliferácie a odolnosti voči apoptóze môžu odhaliť ďalšie vhodné molekulárne ciele pre protinádorovú genovú terapiu malígneho melanómu. Ďalšie diskutované výsledky nesledovali priamo funkciu komplexu SWI/SNF, ale môžu predstavovať cenné poznatky v regulácii transkripčného faktora MITF a pomôcť pochopiť biológiu melanómu. Možná súvislosť s komplexom SWI/SNF je diskutovaná v texte.

V našej práci sme rozpoznali novú úlohu proteínu p21 v melanómových bunkách. Výsledky ukázali, že proteín p21 je v melanómových bunkách prítomný vo vysokých hladinách. Táto hladina je melanómovými bunkami tolerovaná a p21 pozitívne reguluje promótor MITF. Keďže p21 je jedným z transkripčných cieľov pre MITF (Carreira a spol., 2005), pôsobí ako pozitívna spätná väzba a dochádza k vzájomnej aktivácii transkripcie. Vysoké hladiny MITF chránia melanómové bunky pred apoptózou (reostatový model) a možné vysvetlenie je aktivácia expresie antiapoptotického proteínu BCL2 prostredníctvom MITF. K antiapoptotickému potenciálu môže prispievať aj p21, ktorý je v nádorových bunkách lokalizovaný v jadre, ale môže byť i v cytoplasme (nie je ukázané). Zdá sa, že v melanómových líniách p21 neplní funkciu inhibítora bunkového cyklu, ale posiluje antiapoptotický potenciál buniek (súvislosť medzi p21 a Brg1 bola diskutovaná vyššie).

Medzi epigenetické mechanizmy regulujúce chromatínovú štruktúru patria okrem chromatín-remodelujúcich komplexov aj komplexy s histón-acetyltransferázovou aktivitou, ktoré svojou enzymatickou funkciou menia status acetylácie histónov a tým ovplyvňujú prístupnosť promótorových oblastí génov (Khorasanizadeh, 2004). Medzi HAT enzýmy prítomné v takýchto komplexoch patrí aj PCAF a hGCN5, ktoré sú vysoko homológne, ich sekvencia je u eukaryotov na 73% identická (Yang a spol., 1996). Ich expresia v jednotlivých bunkových typoch je však odlišná a rovnako podjednotkové zloženie HAT-komplexov, v ktorých sú obsiahnuté (Nagy a Tora, 2007). V našej práci sme zistili, že majú významný vplyv na aktivitu MITF pri aktivácii jeho cieľových promótorov. Oba fyzicky interagujú s MITF ako rekombinantné proteíny a oba enzýmy inhibujú aktivitu MITF, a to nezávisle na ich histón-acetyltransferázovej aktivite. Ide o celkom nové poznatky a je nutné dokončiť ďalšie pokusy napr. chromatín-imunoprecipitáciu na MITF-cieľových promótoroch a pokusy,

kde bude sledovaný dlhodobý efekt, napríklad hypopigmentácia PCAF-overexprimovaných buniek alebo naopak hyperpigmentácia po zablokovaní expresie PCAF. Výsledky sú pripravované do publikácie a pilotné výsledky sú v prílohe 8 (str. 144). Funkcia týchto enzýmov v bunke je spájaná výlučne s ich existenciou v multi-podjednotkových komplexoch s acetylačnou (príp. ubikvitínligázovou) aktivitou a nie je známe, či PCAF a hGCN5 dokážu operovať v bunke mimo tieto komplexy (Muratoglu a spol., 2003; Demeny a spol., 2007). Korepresorová funkcia na aktivitu MITF by mohla znamenať dosiaľ nepoznanú nezávislú funkciu týchto proteínov.

Medzi signálne dráhy často hyperaktivované v melanómoch (až 60%) patrí dráha PI3K-Akt-mTOR (Gray-Schopfer a spol., 2007). Foster a spol. (2006) zistili priamu interakciu medzi serín/treonín kinázou Akt a podjednotkami SWI/SNF komplexu. INI1 a BAF155 boli schopné priamo interagovať s Akt a v kinázovej reakcii *in vitro* Akt fosforyluje obidva SWI/SNF faktory. V našej práci sme potvrdili vysokú aktivitu dráhy PI3K-Akt-mTOR v niekoľkých melanómových líniách. Už dávnejšie bolo pozorované, že pri zablokovaní cesty mTORC1 prostredníctvom rapamycínu, špecifického inhibítora mTOR, dochádza spätnoväzobným mechanizmom k nárastu aktivity Akt (viď Úvod a literárny prehľad). Tento trend, zablokovanie dráhy mTORC1 s následnou re-aktiváciou Akt, sme po inkubácii s rapamycínom pozorovali aj my v bunkách Hbl.

Signálnu cestu mTORC1 sme ďalej zablokovali pomocou špecifického inhibítora Src kináz SU6656. Src je nereceptorová tyrozín kináza, produkt protoonkogénu c-src a jej zvýšená aktivita bola pozorovaná u značného počtu ľudských nádorov vrátane melanómov (Barnekow a spol., 1987; Homsi a spol., 2007). Dôkazom inhibície cesty mTORC1 bol pokles fosforylácie downstream efektorov mTOR, S6K1 a jej substrátov, ribozomálneho S6 proteínu a proteinu rictor, špecifickej podjednotky komplexu mTORC2. Pokles fosforylácie Akt na S473 predstavujúci pokles jej aktivity je pravdepodobne v dôsledku inaktivácie komplexu mTORC1 zatiaľ neznámym mechanizmom. Prítomnosť Src v imunoprecipitáte raptora, špecifickej podjednotky komplexu mTORC1, potvrdzuje fyzickú interakciu Src s týmto komplexom.

Rictor, špecifická podjednotka komplexu mTORC2, je substrátom pre fosforyláciu kinázou S6K1 (Treins a spol., 2010; Julien a spol., 2010), ktorá je downstream efektorom dráhy mTORC1. Hoci táto fosforylácia rictor na Thr1135 neovplyvňuje aktivitu mTORC2 (Treins a spol., 2010; Julien a spol. 2010), predstavuje prvé prepojenie dráh mTORC1 a mTORC2. V našej práci fosforylácia rictor na Thr1135 klesla po ovplyvnení rapamycínom aj SU6656. A keďže v bunkách ovplyvnených rapamycínom došlo k fosforylácii Akt

na S473, ktorá je substrátom pre fosforyláciu komplexom mTORC2, potvrdila sa nezávislosť aktivity komplexu mTORC2 od fosforylácie rictor v bunkách Hbl.

V súčasnosti sú študované účinky duálnych inhibítorov PI3K/mTOR, ako napr. NVP-BAG956, NVP-BBD130 a NVP-BEZ235, ktoré sa zdajú byť potenciálne efektívnejšie pre terapiu melanómu ako mono-cielené inhibítory (Marone a spol., 2009). Naše pilotné výsledky odhalili možno ďalší vhodný inhibítor v terapii melanómu. Doležitejším sa však zdá byť naše úplne nové zistenie, že Src je v komplexe s mTORC1 v melanómových bunkách a v ďalšej práci je potrebné lepšie objasniť funkčný dopad tejto interakcie.

7. Závery

Výsledky v tejto práci prinášajú nové poznatky v regulácii expresie transkripčného faktora MITF v bunkách malígneho melanómu a umocňujú význam epigenetických mechanizmov v progresii melanómu, predovšetkým úlohu chromatín-remodelujúceho komplexu SWI/SNF. V menšej miere prinášajú nové poznatky v regulácii transkripcie/aktivity MITF mimo SWI/SNF komplex.

- V melanómových bunkách SWI/SNF komplex pozitívne reguluje expresiu MITF. Umlčanie expresie enzýmových podjednotiek Brm a Brg1, ktoré sú podmienkou funkčnosti jednotlivých subkomplexov, výrazne znižuje expresiu MITF a niektorých jeho transkripčných cieľov. Pre prežívanie melanómových buniek a expresiu MITF je nevyhnutná expresia aspoň jednej enzýmovej podjednotky. Umlčanie expresie MITF v melanómových bunkách po 3 dňoch indukuje masívnu apoptózu a inaktivácia SWI/SNF komplexov prostredníctvom knockdownu Brg1 taktiež po 3 dňoch indukuje apoptózu, pravdepodobne v dôsledku umlčanej expresie MITF. Microarray analýza v Brg1-umlčaných bunkách odhalila pokles mRNA dôležitých transkripčných cieľov MITF, známych svojou proproliferačnou funkciou, CDK2 a BCL2. Možno zhrnúť, že funkčný SWI/SNF komplex prispieva k prežívaniu melanómových buniek manipuláciou hladiny transkripčného faktora MITF, kritického líniovo-špecifického faktora melanocytov.
- V porovnaní s normálnymi melanocytmi sú zložky SWI/SNF kopmlexu veľmi dobre exprimované v melanómových bunkách. S výnimkou jednej melanómovej línie SK-MEL-5, ktorá postrádala expresiu Brg1, si melanómové línie zachovávajú expresiu oboch katalytických podjednotiek Brm i Brg1.
- Prioritne dôležitejšími subkomplexami SWI/SNF v expresii MITF v melanómových bunkách sú Brg1-obsahujúce komplexy. Po strate expresie Brg1 môže byť jeho funkcia kompenzovaná funkciou Brm. Striktne zachovaná expresia Brm a heterogénna expresia Brg1 vo vzorkách primárnych melanómov naznačuje, že melanómové bunky si vyvinuli mechanizmus, vďaka ktorému sa po strate Brg1 stáva Brm kriticky dôležitou zostatkovou ATPázou v expresii MITF i v prežívaní melanómových buniek. Príklad tejto kompenzačnej funkcie predstavuje línia SK-MEL-5.
- SWI/SNF komplex prispieva k prežívaniu melanómových buniek aj cestou nezávislou na aktivite MITF, pozitívne reguluje expresiu "prežívacích" faktorov ako napr. IGF1, OPN, TGFß2 a survivín.

- Na základe zistení, že funkčný SWI/SNF komplex prispieva k prežívaniu melanómových buniek mechanizmom závislým i nezávislým na MITF, inaktivácia SWI/SNF komplexu predstavuje sľubný cieľ pre molekulárnu génovú terapiu melanómu.

- V melanómových bunkách sú tolerované vyššie hladiny p21 bez spomalenia proliferácie. p21 pozitívne reguluje transkripciu MITF a spätnoväzobným mechanizmom sa podieľa na udržiavaní hladiny MITF v melanómových bunkách. Tento účinok je nezávislý na funkcii p21 ako inhibítora bunkového cyklu a prispieva k vysvetleniu relatívne vysokej expresie p21 v melanómových bunkách.
- Histón-acetyltransferázy PCAF a hGCN5 pôsobia ako korepresory transkripčného faktora MITF a inhibujú jeho aktivitu na jeho cieľovom, melastatinovom promótore nezávisle na ich histón-acetyltransferázovej aktivite. Tieto výsledky naznačujú nový, doteraz nepoznaný mechanizmus regulácie aktivity MITF v melanómových bunkách.
- K progresii malígneho melanómu prispievajú hyperaktivované signálne dráhy a k takýmto patrí signálna cesta PI3K-Akt-mTOR. Špecifický inhibítor Src-kinázovej aktivity inhibuje aktivitu mTORC1 komplexu, podobne ako špecifický inhibítor mTOR, rapamycín. Na rozdiel od rapamycínu, ktorý spôsobuje reaktiváciu Akt spatnoväzobným mechanizmom, SU6656 inhibuje fosforyláciu Akt na S473 a tým prispieva k jej deaktivácii. SU6656 predstavuje sľubný nástroj pre cielené umlčanie PI3K-dráhy v melanómových bunkách a ďalšie štúdium jeho účinku by mohlo odhaliť možné uplatnenie v terapii melanómu.

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9. Prílohy

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Biochemical and Biophysical Research Communications 392 (2010) 454–459

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

SWI/SNF chromatin remodeling complex is critical for the expression of microphthalmia-associated transcription factor in melanoma cells

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ARTICLE INFO

Article history:
Received 12 January 2010
Available online 18 January 2010

Keywords:
SWI/SNF complex
MITF
Microphthalmia
Melanoma
Brg1

ABSTRACT

The microphthalmia-associated transcription factor (MITF) is required for melanocyte development, maintenance of the melanocyte-specific transcription, and survival of melanoma cells. MITF positively regulates expression of more than 25 genes in pigment cells. Recently, it has been demonstrated that expression of several MITF downstream targets requires the SWI/SNF chromatin remodeling complex, which contains one of the two catalytic subunits, Brm or Brg1. Here we show that the expression of MITF itself critically requires active SWI/SNF. In several Brm/Brg1-expressing melanoma cell lines, knockdown of Brg1 severely compromised MITF expression with a concomitant dowregulation of MITF targets and decreased cell proliferation. Although Brm was able to substitute for Brg1 in maintaining MITF expression and melanoma cell proliferation, sequential knockdown of both Brm and Brg1 in 501mel cells abolished proliferation. In Brg1-null SK-MEL-5 melanoma cells, depletion of Brm alone was sufficient to abrogate MITF expression and cell proliferation. Chromatin immunoprecipitation confirmed the binding of Brg1 or Brm to the promoter of MITF. Together these results demonstrate the essential role of SWI/SNF for expression of MITF and suggest that SWI/SNF may be a promissing target in melanoma therapy.

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Introduction

Mammalian SWI/SNF complex is a chromatin remodeling machinery present in cells as several subcomplexes each consisting of 10–12 protein components and having only subtle differences in subunit composition [1–3]. SWI/SNF complexes, when recruited to DNA by the DNA-binding transcription factors, can either activate or repress transcription by altering the nucleosome structure or assembly at the promoter regions. The energy for chromatin remodeling is provided by either Brm (SMARCA2) or Brg1 (SMARCA4), two enzymes with ATPase activity which are present in the complexes in a mutually exclusive manner. The BAF subcomplex can be assembled with either ATPase and has a diagnostic subunit BAF250, whereas the PBAF subcomplex contains Brg1 and is specified by the presence of BAF180 and BAF200 [1,3]. Other subunits are shared by all complexes and include BAF47/INI1, BAF53, BAF57, actin, BAF60, BAF155, and BAF170.

Loss of several SWI/SNF subunits has been reported in malignant cells implicating the SWI/SNF complex in tumor suppression [1,3]. A number of reports have demonstrated that Brg1 or Brm are lost, either individually or concomitantly, in about 20–30% of cancer cell lines derived mostly from non-small cell lung cancer. Lack of expression was revealed also in tumor tissue, and the loss of Brm or Brg1 was implicated in cancer progression [1,4–7]. However, some cancer cells such as gastric or prostate have aberrantly increased expression of Brg1 [8,9]. In the prostate, the SWI/SNF complex, and specifically its BAF57 subunit, is required for the activity of androgen receptor, a transcription factor critical for proliferation of androgen-dependent prostate carcinomas [10,11]. Thus, SWI/SNF may behave also as a tumor promoter, depending on the tissue context.

Melanocytes are cells derived from the neural crest and the basic-helix-loop-helix protein MITF-M (melanocyte-specific isoform of MITF, referred to as MITF herein) plays an essential role in their embryonic development, maintenance of lineage identity, and differentiation. Importantly, MITF is also essential for the survival of melanoma cells and has been considered as a melanoma oncogene [12]. MITF targets are genes involved in pigment formation, cell cycle regulation (p21 and CDK2) [13,14], apoptosis (bcl-2 and livin) [15,16] and organization of cytoskeleton (Dia1) [17], among others [18]. In the zebrafish, Brg1-mutated embryos revealed defects in the development of pigment cells and retina, the tissues expressing MITF [19]. Expression of several pigment cell-specific MITF targets was recently reported to be dependent on SWI/SNF chromatin remodeling complex. It has been shown that MITF interacts with ATPases Brg1 or Brm to activate targets such as pigmentation

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doi:10.1016/j.bbrc.2010.01.048

related genes (Tyr, TRP-1, and Dct), cdk2, livin, and some others [20,21]. In this study, we demonstrate that SWI/SNF acts upstream of MITF and is critically involved in the expression of MITF itself, further implicating SWI/SNF as a transcription coactivator important for survival and proliferation of melanoma cells.

Materials and methods

Cell culture. 501mel cells were gift from Dr. R. Halaban (Dept. Dermatol, Yale University) and maintained in the RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. Other melanoma cell lines were obtained and cultured as described [22,23]. H1299 (ATCC) and 293FT cells (Invitrogen) were maintained in DMEM with 10% FBS and antibiotics.

Plasmids and transfections. Human melanocyte-specific MITF promoter was cloned by inserting the regulatory region (nucleotides −2878 to +104) in the pGL3 basic (Promega). The promoter-luciferase was also recloned into the pREP4 plasmid (Invitrogen). Plasmid pBJ5-Brg1 was provided by Dr. G. Crabtree (Stanford University). The K785R Brg1 mutant was generated by QuickChange mutagenesis kit (Stratagene). pCG-Brm and CG-Brm-ATPmut plasmids were provided by Dr. C. Muchardt (Institut Pasteur, Paris). Cells were transfected using Lipofectamine 2000 (Invitrogen) and either selected in 2.0 μg/ml puromycin or assayed for firefly and Renilla luciferase activity using the Dual Luciferase Reporter Assay reagents (Promega).

Short hairpin RNA knockdowns and generation of lentiviruses. The shRNA sequences were cloned in the pSUPER.puro vector (Oligoengine). The target sequence for knockdown of human MITF was GGACAATCACAACCTGATTG. The Brm, Brg1, and the common Brm/Brg1 target sequences were described previously [24], and are as follows: Brm, GGAGGTGCTAAGACACTTATG; Brg1, GTAGCT CCGAGGTCTGATAG; common Brm/Brg1, GCTGGAGAAGCAGCA-GAAG. The non-targeting sequence was GACGGTACCGCGAAC-GaGC. To generate lentiviral vectors, EcoRI-ClaI fragments containing the hairpin were recloned from pSUPER.puro into pLVTHM (Addgene), expressing also GFP [25]. The lentiviruses were produced by cotransfecting 293FT cells with the shRNA-containing pLVTHM plasmid, the pMD2.G, and psPAX2. We used virus titers that infected more than 98% of cells (as assessed by GFP positivity). Cells were infected in the presence of 8 μg/ml polybrene for 48 h, replated, and harvested for analyses 3–6 days later.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were carried out with the EZ-ChIP kit (Upstate). Antibodies used were anti-Brm (Abcam), anti-Brg1 (G-7, Santa Cruz), and anti-acetyl histone H4 (Upstate). Primers for amplification of the proximal MITF promoter region spanning its regulatory sites were 5′-TCGGCCATTGGAAAAGTCTC and 5′-TCCAGCATTTC-CAGCATAACA. Control primers that amplify a non-related GAPDH promoter region were from Upstate. CHIP was quantified by the QuantiFast SYBR green PCR kit (Qiagen).

RT-PCR and real-time PCR. Isolation of RNA and RT-PCR was carried out as described [26]. The real-time PCR primers for human tyrosinase were 5′-CCAGAAGCTGACAGGAGATG and 5′-AGG-CATTGTGCATGCTGCTT. The probe for human tyrosinase was 5′-6-FAM-ACGGCGTAATCCTGGAAACCATGACA-TAMRA. To quantitate MITF transcripts, the primers and Taqman probe was used as described by Dynek et al. [16]. To quantitate CDK2 and melastatin transcripts, primers and probes were used as described [14,27]. β-Actin primers used in control amplifications were 5′-ATTGCCGA-CAGGATGCAGAA and 5′-GCTGATCCACATCTGCTGGAA. The probe for β-actin was 5′-6-FAM-CAAGATCATTGCTCCTCCTGAGCGCA-TAMRA. Quantitect Probe PCR Kit (Qiagen) was employed for real-time PCR reactions as recommended by the supplier and the reaction products were detected with an ABI Prism 7700 instrument (Applied Biosystems). Expression values were normalized to values of β-actin.

Immunoblotting. Whole cell or nuclear extracts [23] were subject to Western blot analysis. Primary antibodies purchased from Santa Cruz were: Brg1(G-7), BAF155(H-76), BAF170(H-116), BAF200(2036C1a), BAF250A(PCG3). Antibodies against Brm, BAF53a, and BAF57 were purchased from Abcam. Antibodies to INI1 and BAF60a were from BD Biosciences. Anti-MITF antibody (D5) was from Labvision, and anti-β-actin from Sigma.

Colony formation assays and cell proliferation. For proliferation assays, transfected or infected cells were seeded in triplicate wells, grown for appropriate time intervals, fixed and stained with 0.1% crystal violet, and the dye was quantified. For colony formation assays, cells were replated two days after transfection into the puromycin-containing medium, and fixed, stained and quantified after 12 days.

Statistical methods. Statistical analyses of proliferation rates, real-time RT-PCR, and luciferase assays were done using Student's *t*-test.

Results

Expression of SWI/SNF components in melanoma cell lines

Nuclear extracts from melanoma lines, together with normal human melanocytes (HeMn), were immunoblotted. Western blot revealed a generally higher expression of most SWI/SNF components in eight melanoma cell lines, whereas low levels of some BAFs (BAF 53a, BAF57, and BAF200) were observed in primary melanocytes (Fig. 1). Five additional melanoma cell lines derived from metastatic tissue (HT144, Beu, DOR, LND1, and SK-MEL-1) were analyzed and, likewise, a complete expression of SWI/SNF subunits was revealed (not shown). Interestingly, only one cell line, SK-MEL-5 showed a loss of Brg1 protein, as also reported recently [21]. Thus, the expression of SWI/SNF proteins in melanoma lines is

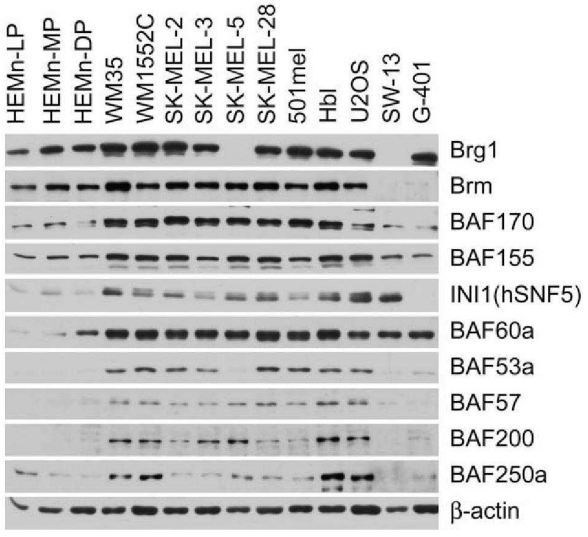

Fig. 1. SWI/SNF subunits expression in human melanoma cell lines and normal melanocytes. Protein expression was determined by immunoblotting of extracts from lightly, moderately, and darkly pigmented human skin melanocytes (HEMn-LP, -MP, and -DP), two primary melanoma cell lines (WM35 and WM1552C), and six metastasis-derived cell lines (SK-MEL-2, SK-MEL-3, SK-MEL-5, SK-MEL-28, 501mel, and Hbl). Additional five melanoma cell lines displayed complete expression of all subunits (not shown, see Results). U2OS cells (positive control) expressed all subunits, SW13 cells are negative for Brg1 and Brm [7], and G-401 is INI1-negative cell line.

generally well preserved or even increased when compared with normal melanocytes.

Brg1 and Brm activate the promoter-reporter and associate with MITF promoter in vivo

Transcription from the melanocyte-specific promoter of MITF is driven by activators Pax3, Sox10, CREB, and Wnt-β-catenin pathway efectors TCF/LEF [18]. Brg1 has been previously shown to interact with β-catenin to activate TCF target genes [28], suggesting a possible involvement of Brg1 in MITF transcription. Expression vectors for Brm and Brg1, or their dominant-negative (DN) ATPase-null mutants, were transfected with the MITF promoter-reporter into SK-MEL-5 cells. As shown (Fig. 2A, left), co-transfection of either Brm or Brg1 increased the promoter activity, whereas both DN-mutants were slightly inhibitory. Even more pronounced inhibition was seen with DN-mutants when the promoter was cloned in the pREP4 plasmid, which is known to form episomal nucleosome-like structures resembling endogenous promoters, whereas the promoter responded only insignificantly to wt proteins in this case (Fig. 2A, middle). This is consistent with the notion that exogenously increased level of Brm or Brg1 cannot further elevate the expression from an endogenous promoter, as SWI/SNF subunits are present in stoichiometric amounts in the cell. No effect of Brm or Brg1 vectors was seen on the basic promoter (Fig. 2A, right); Western blot shows expression of Brm or Brg1 (see legend to Fig. 2).

Next, we performed a chromatin immunoprecipitation (ChIP) assay to examine the recruitment of the two ATPases onto the MITF promoter in vivo. The results (Fig. 2B) revealed Brm bound to the proximal region of the MITF promoter in SK-MEL-5 cells. By contrast, 501mel and Hbl cells displayed only Brg1 occupancy. Amplification of a control promoter showed no specific signal pulled by the anti-Brg1 or anti-Brm antibodies (Fig. 2B).

Quantitation of CHIP confirmed high enrichment of Brm on the MITF promoter in SK-MEL-5 cells (14-fold). The presence of Brg1

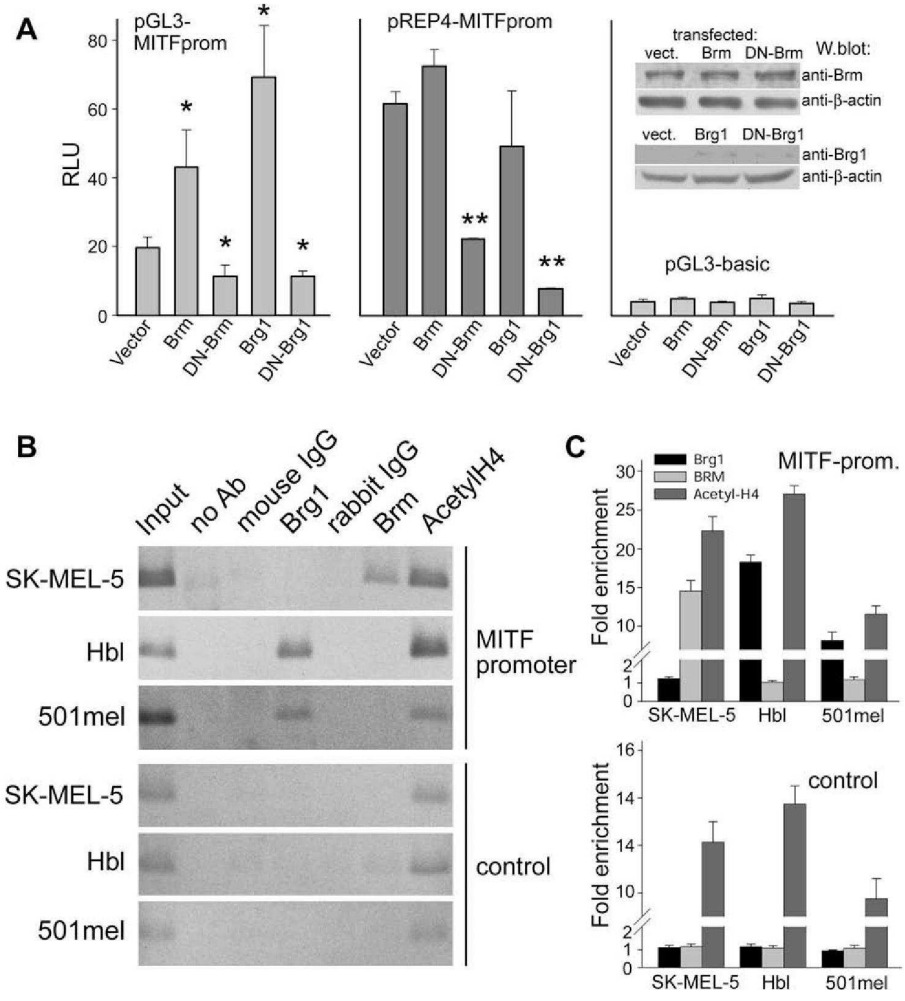

Fig. 2. Brg1 or Brm binds to MITF promoter in ChIP assays and MITF promoter-reporter activity is repressed by cotransfected DN-mutants. (A) The effect of Brg1, Brm, and their DN-mutants on MITF promoter-reporter in SK-MEL-5 cells and on the control promoter (pGL3basic). Insert (right): expression of Brm and Brg1 detected by a Western blot. Note that SK-MEL-5 cells express Brm but not Brg1 and because only a small fraction of cells is transfected, the change of the Brm signal is minimal. All four expression vectors are functional as revealed in reporter assays and further checked by transfection into Brm- or Brg1- negative cells (not shown). Asterisks show statistical significance of values compared with samples transfected with vector ($^{*}P < 0.05$; $^{**}P < 0.01$). RLU, relative luciferase activity (normalized to Renilla luciferase values) of duplicate samples + SE. (B) Brg1 or Brm binds to the endogenous MITF promoter in melanoma cells. Chromatin immunoprecipitations from SK-MEL-5, 501mel and HBl cells are shown. Control amplification detected the GAPDH promoter region. (C) Quantification of DNA in the chromatin immunoprecipitation reactions detected by primers for the MITF promoter (upper panel) or control region (lower panel). Fold enrichment denotes the increase of Brm, Brg1, and acetyl histone H4 immunoprecipitated by specific antibodies in comparison with corresponding control IgG.

but not Brm on the promoter was increases in Hbl (18-fold) and 501mel cells (8-fold), while no binding of both ATPases was seen on the control promoter (Fig. 2C). Thus, the ChIP results suggest that Brg1 might be the primary ATPase recruited to MITF promoter in Brm/Brg1-positive melanoma cells while Brm occupies the promoter if Brg1 is lost. Together the reporter and ChIP data imply that Brg1 or Brm may function as cofactors for transcription of MITF in melanoma cells.

Brg1 depletion downregulates MITF expression

To further elucidate a link between Brg1 and Brm levels and MITF expression, shRNA constructs were used to specifically deplete Brg1 or Brm in melanoma cell lines. Several shRNAs were first tested for effectiveness and one shRNA for each enzyme was selected for experiments. Further, a sequence shared by Brg1 and Brm [24] was also targeted to deplete both proteins simultaneously. As shown in Fig. 3A, MITF protein expression was dramatically reduced in the Brg1-depleted 501mel cell line, whereas no effect or only a mild decrease was seen after the depletion of Brm (experiments 1 and 2, Fig. 3A). Simultaneous Brm/Brg1 silencing was less efficient for Brg1 and resulted in a significant but incomplete MITF knockdown. In MeWo cells, depletion of Brg1 or Brm decreased the MITF protein (Fig. 3B). Targeting Brm alone was sufficient to repress MITF expression in SK-MEL-5 cells, (Fig. 3C). Taken together, the data implicate the SWI/SNF complex as a critical factor for MITF expression with Brg1 being clearly the primary cofactor in SWI/SNF-intact 501mel melanoma cells.

To provide more direct evidence that Brg1 or Brm regulates transcription of endogenous MITF, we determined the transcript levels in Brg1-shRNA and Brm-shRNA-transfected 501mel cells by real-time PCR. Levels of mRNA for MITF and its target genes melastatin, CDK2, and tyrosinase were substantially reduced upon Brg1 depletion, whereas the levels decreased only ~2-fold in Brm-silenced cells (Fig. 3D), confirming the primary role of Brg1 for expression of MITF and its targets in 501mel cells.

Knockdown of both Brm and Brg1 abrogates proliferation of 501mel cells

High expression of MITF has been associated with proliferation and survival of melanoma cells via its target genes CDK2, Bcl-2, livin, c-met, and HIF1α [14–16,29,30]. We therefore determined proliferation rates in Brg1, Brm, and Brg1/Brm-depleted cells. As expected, SK-MEL-5 cells in which Brm expression was inhibited stopped proliferating (Fig. 4A). MeWo cells, however, were only partially growth-inhibited by sh-Brm, sh-Brg1, or sh-Brm/Brg1-expressing viruses, consistent with the observed contribution of both ATPases to MITF expression in these cells (Fig. 3B). In infected 501mel cells, the initial growth inhibition was surprisingly overcome and cells resumed proliferation about 6 days after reseeding (Fig. 4A, right).

Because the 501mel cells transduced with the sh-Brg1-expressing lentivirus resumed proliferation after an initial growth inhibition, we determined whether Brg1 was stably extinguished in these cells. The expression of Brg1 remained efficiently blocked

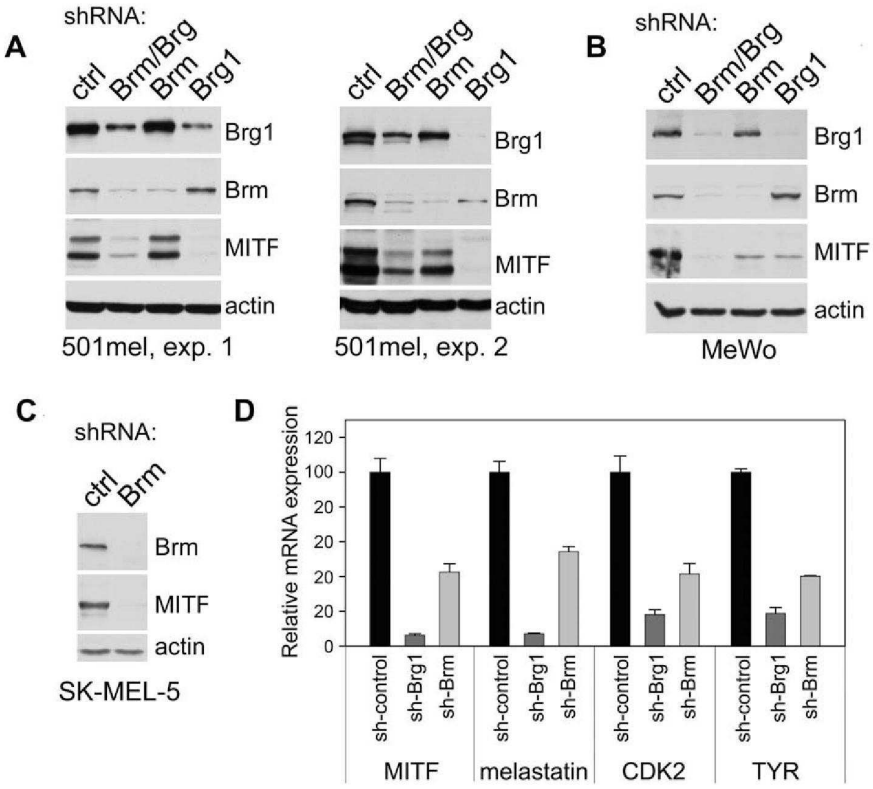

Fig. 3. SWI/SNF complex is required for MITF expression in melanoma cells. 501mel and MeWo cells were transfected with shRNA plasmids and SK-MEL-5 cells were infected with lentiviruses. (A) Brg1 is necessary for MITF expression in 501mel cells (experiments 1 and 2). (B) depletion of Brm or Brg1 in MeWo cells attenuates MITF expression. (C) Depletion of Brm represses MITF expression in SK-MEL-5 cells. (D) Real-time PCR experiment showing downregulation of MITF and its targets TYR, melastatin, and CDK2 after knockdown of Brg1 or Brm in 501mel cells. All real-time PCR values for sh-Brg1 and sh-Brm-treated cells were statistically significant ($P < 0.01$) when compared with controls. Bars, +SE of triplicate samples.

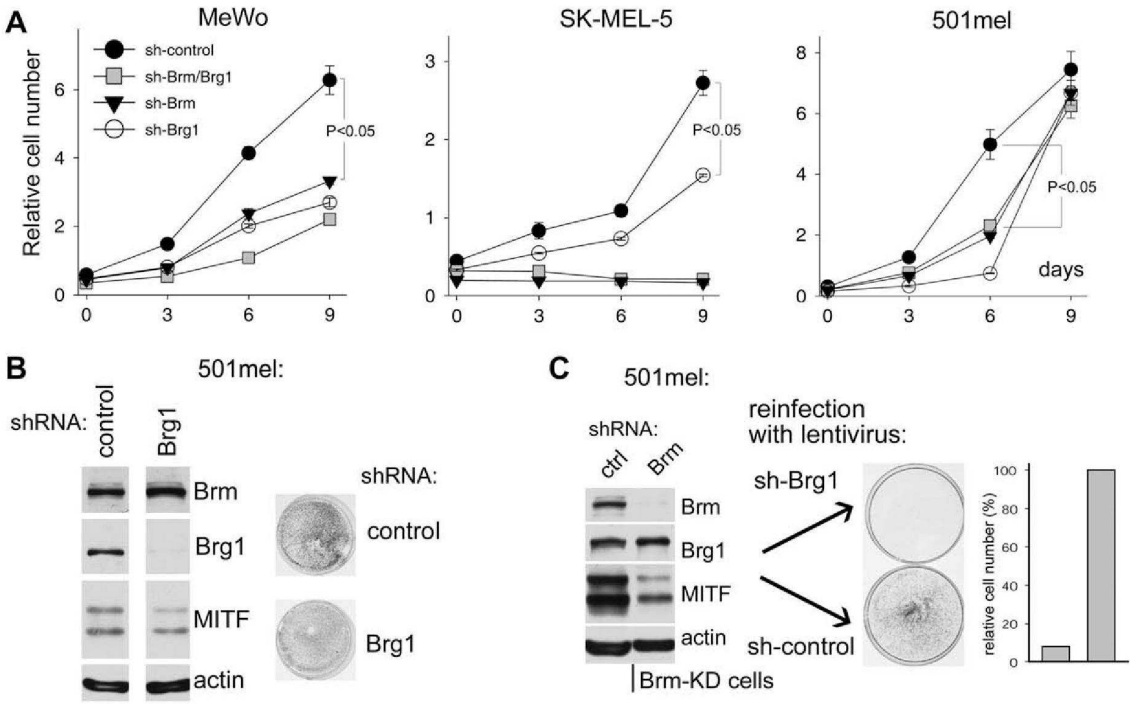

Fig. 4. Growth rates of melanoma cells after knockdown of Brg1 or Brm expression and sequential knockdown of both ATPases. (A) Growth rates of MeWo, SK-MEL-5, and 501mel cells after depletion of Brg1, Brm, or both. Points, means of triplicate samples; bars, SE. Note that sh-Brg1 slightly nonspecifically retarded growth of SK-MEL-5 cells after a longer time (at day 9). (B) Brm is able to compensate for Brg1 loss in 501mel cells. Brg1-depleted cells express MITF and continue proliferation. (C) Brg1 knockdown in Brm-depleted 501mel cells (Brm-KD) causes cessation of cell growth.

10 days after infection with sh-Brg1 lentivirus and Brm protein was fully expressed (Fig 4B, left). Correspondingly, colony growth was not suppressed. Thus, evidently, Brm can compensate for Brg1 loss.

Given that Brm alone was sufficient to activate MITF expression and sustain proliferation, we next examined the effect of sequential knockdown of both Brm and Brg1. To this end, Brm was first depleted in 501mel cells (Fig. 4B, right) and then the cells were reinfected by either control or sh-Brg1-expressing virus. Clearly, almost no Brm-depleted cells survived this Brg1 knockdown while scrambled shRNA-infected cells continued proliferating (Fig. 4B, right). These results together indicate that Brm is able to compensate for Brg1 depletion and at least one ATPase must be expressed in order for melanoma cells to express MITF and proliferate.

Discussion

In this study, we provide data indicating that the core ATPases of the SWI/SNF chromatin remodeling complex function as essential cofactors of MITF expression in melanoma cells. We showed that Brg1 (or Brm in Brg1-null melanoma cells) is present at the MITF promoter in vivo, indicating that local chromatin remodeling is necessary for the activity of the melanocyte-specific MITF promoter. Brg1 knockdown efficiently depleted MITF in all melanoma cell lines expressing Brg1 and Brm. Although our results suggest that Brg1-containing complexes could preferentially mediate MITF expression, Brm can readily act as a surrogate enzyme if Brg1 expression is lost. This situation evidently occurred in SK-MEL-5 cell line and in Brg1-depleted 501mel cells. In contrast to MITF expression observed here, Brm and Brg1 may be more strictly specialized to coactivate downstream MITF target genes [21]. A compensatory role of Brm for Brg1 loss has been described previously in other cell systems; for example, Brm was able to restore the Rb-dependent sensitivity to cell cycle arrest in Brg1/Brm-defficient cells [31] or coactivate induction of p21 in Brg1-knocked down MCF7 cells [32].

In a recent study utilizing a different panel of melanoma cell lines, two cell lines revealed loss of Brm protein [21]. By contrast, we observed here that Brm was expressed in all melanoma cell lines analyzed. Interestingly, Becker et al. [33] recently described undetectable Brg1 protein in more than a half of primary and metastatic melanomas while Brm negativity was seen only in ~20% of cases. Thus, conceivably, Brm might become a critical remaining ATPase maintaining the expression of MITF in melanoma cells in vivo under conditions of Brg1 unavailability.

Several transcription factors participate in the activation of the MITF gene in melanocytes, including the Wnt/β-catenin pathway effectors TCF/LEF [18]. Because β-catenin-induced growth of melanomas requires MITF [34], and β-catenin interacts with Brg1 to promote activation of TCF/LEF targets [28], the TCF/LEF proteins might be important for recruitment of SWI/SNF to activate MITF expression.

Our RNA silencing experiments further indicate that melanoma cells exert defects in proliferation when both Brg1 and Brm are depleted, which could be attributed to the loss of MITF expression. However, because the expression of proliferation-related downstream targets such as cdk2 and livin were shown to be also SWI/SNF-dependent [21], the effect of inhibition of SWI/SNF activity on proliferation may be combined. Moreover, as melanoma lines were found to be differentially sensitive to MITF depletion [35], MITF target genes may play distinct roles in survival of individual cell lines.

Recently, Brg1 has been shown to destabilize the p53 protein by binding to both p53 and coactivator CBP, resulting in increased p53 ubiquitination and degradation with consequent decreased p21 level [36]. Although we observed slightly elevated p53 levels in

Brg1-, and even MITF-depleted 501mel cells, no upregulation of p21 protein level was seen (data not shown). Moreover, cell lines which harbor mutated p53 (MeWo and SK-MEL-28) also exerted defects in proliferation and viability (Fig. 4A, and data not shown). It is therefore unlikely that attenuation of cell proliferation was due to p53 activation.

In summary, we have shown that the inactivation of SWI/SNF complex results in a profound downregulation of MITF expression in melanoma cells which subsequently affects expression of downstream genes and proliferation of melanoma cells. Thus, SWI/SNF complex is not only a coactivator of expression of many MITF target genes, but it is also an essential cofactor for transcription of MITF itself in melanoma cells. Our data suggest that a tissue-aimed inactivation of the SWI/SNF complex might become an effective approach in the therapy of melanoma.

Acknowledgments

We thank E. Křepela for help with real-time PCR experiments, H. Novotná and J. Réda for technical assistance, and Č. Vlček for DNA sequencing. We also thank R. Halaban for 501mel cells and C. Muchardt and G. Crabtree for kindly providing the Brm and Brg1 expression constructs, respectively. This work was supported by Grant NR/9319-3 from IGA MZ, Czech Republic.

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MITF-Independent Pro-Survival Role of BRG1-Containing SWI/SNF Complex in Melanoma Cells

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Abstract

Metastasized malignant melanoma has a poor prognosis because of its intrinsic resistance to chemotherapy and radiotherapy. The central role in the melanoma transcriptional network has the transcription factor MITF (microphthalmia-associated transcription factor). It has been shown recently that the expression of MITF and some of its target genes require the SWI/SNF chromatin remodeling complex. Here we demonstrate that survival of melanoma cells requires functional SWI/SNF complex not only by supporting expression of MITF and its targets and but also by activating expression of prosurvival proteins not directly regulated by MITF. Microarray analysis revealed that besides the MITF-driven genes, expression of proteins like osteopontin, IGF1, TGFß2 and survivin, the factors known to be generally associated with progression of tumors and the antiapoptotic properties, were reduced in acute BRG1-depleted 501mel cells. Western blots and RT-PCR confirmed the microarray findings. These proteins have been verified to be expressed independently of MITF, because MITF depletion did not impair their expression. Because these genes are not regulated by MITF, the data suggests that loss of BRG1-based SWI/SNF complexes negatively affects survival pathways beyond the MITF cascade. Immunohistochemistry showed high expression of both BRM and BRG1 in primary melanomas. Exogenous CDK2, osteopontin, or IGF1 each alone partly relieved the block of proliferation imposed by BRG1 depletion, implicating that more factors, besides the MITF target genes, are involved in melanoma cell survival. Together these results demonstrate an essential role of SWI/SNF for the expression of MITF-dependent and MITF-independent prosurvival factors in melanoma cells and suggest that SWI/SNF may be a potential and effective target in melanoma therapy.

Citation: Ondrušová L, Vachtenheim J, Réda J, Žáková P, Benková K (2013) MITF-Independent Pro-Survival Role of BRG1-Containing SWI/SNF Complex in Melanoma Cells. PLoS ONE 8(1): e54110. doi:10.1371/journal.pone.0054110

Editor: Fatah Kashanchi, George Mason University, United States of America

Received July 4, 2012; **Accepted** December 10, 2012; **Published** January 17, 2013

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Funding: This work was supported by a grant no. NT/11229-3 (to JV) from the Internal Granting Agency, Ministry of Health (www.mzcr.cz), Czech Rep. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Co-author Jiri Vachtenheim is a PLOS ONE Editorial Board member, because he served as a reviewer of one PloS ONE article. This does not alter our adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

Malignant melanoma is highly invasive and early metastasizing tumor, and its incidence has been increasing in recent years [1,2]. In the melanocyte lineage, MITF-M (melanocyte-specific isoform of MITF, referred to as MITF in the text), a member of the large basic helix-loop-helix leucine zipper family of transcription factors, plays an essential role in the embryonic development, maintenance of lineage identity, and differentiation. MITF is central for the transcription of genes involved in various cellular processes from embryonic development of melanocytes to metastasis of melanoma [3,4,5]. Targets of MITF include genes involved in pigment formation [5], cell cycle regulation (p21 and CDK2) [6,7], apoptosis (Bcl-2 and livin) [8,9] and organization of cytoskeleton (diaphanous-related formin Dia1) [10]. MITF expression is heterogeneous in advanced melanomas [11] but is highly expressed at the early phases of melanocyte transformation.

SWI/SNF chromatin remodeling complexes are consisting of about 12 proteins, and are present in cells as several subcomplexes having only subtle differences in subunit composition [12,13,14]. They alter the local nucleosome structure at the promoter regions to regulate transcription. These complexes use the energy provided by either BRM (Brahma, SMARCA2) or BRG1 (Brahma-related gene, SMARCA4), two homologous enzymes with ATPase activity which are present in the complexes in a mutually exclusive manner [14]. The complexes containing BRG1 or BRM may have distinct specificity toward different promoters or may function promiscuously depending on cell and promoter context.

Two subunits of the SWI/SNF complex, INI1/hSNF5/BAF47 and BRG1, are regarded as tumor suppressors. The INI1/hSNF5 subunit is a bone fide tumor suppressor whose homozygous inactivation results in rhabdoid tumors in humans [15]. A number of reports have demonstrated that BRG1 or BRM are downregulated or inactivated in cancer cell lines and tumor samples derived mostly from non-small cell lung cancer. BRG1 has been described frequently mutated in lung cancer cell lines [16]. In contrast to BRG1, BRM is inactivated by epigenetic mechanisms [17]. Loss of BRM or BRG1 was implicated in cancer progression [14,17,18,19]. This was partly attributed to the necessity of BRG1 in Rb-mediated cell cycle arrest [20,21]. However, the function of SWI/SNF is controversial because some cancer cells such as from gastric or prostate tumors have aberrantly increased expression of BRG1 [22,23]. Thus, SWI/SNF can behave also as a tumor promoter, depending on the cancer tissue context.

Expression of MITF and several pigment cell-specific MITF target genes have been previously reported to be dependent on SWI/SNF chromatin remodeling complex [24,25,26]. It has been shown that expression of dominant-negative (DN) mutants of BRM and BRG1 repressed transcription of melanocyte markers (tyrosinase, Trp1, dct, and silver), the expression of which was induced by exogenous MITF in murine fibroblasts [24]. In human melanoma cell lines, many MITF targets require the SWI/SNF complex for expression. As for MITF expression, either BRG1 or BRM can be present in the complex [25,26,27] to activate transcription of MITF targets. Moreover, BRG1 expression was found increased in primary melanoma and metastatic melanoma when compared with dysplastic nevi and reduction of BRG1 expression by siRNA in melanoma cell lines resulted in a significantly decreased cell proliferative ability [28], concordant with our previous results [26] and findings presented here. Furthermore, BRG1 expression has been reported to be elevated during melanoma progression and modulated the expression of some of the extracellular matrix and adhesion proteins [29] such as the metastasis-associated protein metalloproteinase (MMP) 2, which contributed to the BRG1-mediated increase of melanoma invasiveness [29].

In the present work, we identify novel proteins repressed by the inactivation of BRG1 expression, which are not directly regulated by MITF. Importantly, we find here that several additional pro-survival proteins (osteopontin, IGF1, TGFß2, and survivin) are downregulated after the acute depletion of BRG1, together implicating SWI/SNF as a transcription coactivator important for survival and proliferation of melanoma cells. We also demonstrate that the BRG1 knockdown suppressed proliferation of melanoma cells and both BRG1 and BRM are highly expressed in melanoma samples. These results indicate that BRG1 is involved in melanoma progression and possibly metastasis through both the MITF-dependent and independent mechanisms and suggest the disparity of the SWI/SNF function in different types of cancer cells.

Results

Ablation of SWI/SNF Function by the BRG1 Knockdown in 501mel Melanoma Cells Inhibits Proliferation, Decreases Viability and Induces Apoptosis

Acute silencing of MITF causes cell cycle arrest in 501mel cells [10]. Consistently, both sh-BRG1 and sh-MITF-transfected cells revealed diminution of cell numbers in the S/G2 phases of the cell cycle after two days following puromycin selection (Figure 1A, left panels). However, when cells were analyzed after additional 3 days in culture, enhanced cell detachment was observed, accompanied by a prominent increase of cell counts in the sub-G1 phase, indicating apoptosis (Figure 1A, right panels). Apoptotic nuclei were observed in the remaining adherent cells in sh-MITF and sh-BRG1 cells but not in controls (Figure S1). In addition, detached and remaining attached cells were separately analyzed by flow cytometry and high sub-G1 and G1 contents were observed for detached cells (Figure S2) while no G2/M and very low S phase of the cell cycle were dectected, indicating that detached cells did not proliferate and most of them succcumbed to apoptosis. To further document the apoptotic process appearing 3 days after puromycin selection, we detected apoptosis by TUNEL assay (Figure S3). In this case, more prominent apoptosis was seen in sh-MITF than in sh-BRG1 cells. This might be due to a more immediate effect of MITF (which directly activates many apoptosis-inducing genes), while the apoptosis caused by silencing of BRG1 may require more time because MITF expression has to be inhibited first. No apoptosis was detected in sh-control cells. Expression of both BRG1 and MITF was effectively blocked by appropriate shRNA, as assessed by Western blots (Figure 1A, right). However, only slight activation of caspase 3 and 7 and no PARP cleavage was revealed (not shown), implying that cell death in sh-BRG1 and sh-MITF-transfected 501mel cells was predominantly caspase-independent. Similar caspase-independent triggering of apoptosis has also been observed in melanoma cells after the inactivation of survivin function by its dominant negative mutant [30].

Expression of MITF has been associated with proliferation and survival of melanoma cells via its target genes CDK2, Bcl-2, livin, c-met, and HIF1α [6,8,9,31,32]. Indeed, both BRG1 or MITF depletion severely reduced proliferation of 501mel cells, whereas the growth of BRG1/BRM non-expressing A427 lung carcinoma cells (used as a control to exclude a possible off target effects of sh-BRG1) was not inhibited, indicating that the effect of sh-BRG1 plasmid was specific (Figure 1B, upper, left). Depletion of BRM in BRG1-negative SK-MEL-5 cells [26] was sufficient to block proliferation, whereas the growth of BRG1/BRM-expressing RPMI7951 melanoma cells, which are exceptionally MITF-negative [33], was not affected (Figure 1C). Western blots (Figure 1B right) show efficient inhibition of BRG1 or MITF expression in 501mel cells, or BRM in SK-MEL-5 cells.

Further, cell viability was negatively impacted to various degree upon knockdown of BRM, BRG1, or both, in MeWo, Beu and 501mel cells, while only BRG1-negative SK-MEL-5 cells became less viable after BRM-depletion alone (Figure 2). The highest decrease of viability in cell lines expressing both BRG1 and BRM was seen when both were depleted concomitantly by using the common BRG1/BRM shRNA. All these results are in line with the necessity that at least one ATPase must be present for proliferation and viability of each cell line [26]. Cell viability was not affected in A427 cells used as controls because they do not express either BRG1 or BRM.

Microarray Analysis of Gene Expression after BRG1 Knockdown in 501mel Cells

We next used cDNA microarrays to investigate the transcriptional response to the BRG1 depletion in a genome-wide analysis (Figure 3A). We chose the BRG1 knockdown, because its depletion resulted in much stronger inhibition of MITF and its three analyzed target genes (melastatin, CDK2, and tyrosinase) than ablation of BRM in 501mel cells, implicating that BRG1-based SWI/SNF complexes may play more important role in expression of these and possibly other target genes [26]. The changes in gene expression were studied shortly after the complete inhibition of BRG1 (2 days after a 2-day selection in puromycin), ensuring that the changes in expression profile were due exclusively to BRG1 loss. In the microarray analysis, we found that expression of 381 genes (represented by 485 probes) had been downregulated >2-fold ($P<0.005$) (Table S1), including some of the known MITF targets (Table S2), while other MITF targets were decreased to a lesser extent (not shown). Sox10, the upstream MITF transactivator, was also repressed, by ~5-fold (Table S1). By contrast, a smaller number of genes (210, represented by 273 probes) was upregulated >2-fold ($P<0.005$) (Table S3). Interestingly, among the strongly repressed genes (67 genes, downregulated >8-fold, $P<0.005$; Figure 3A and Table S1) are insulin-like growth factor 1 (IGF1) and osteopontin (OPN, also named SPP1), two factors known to have anti-apoptotic, proliferative, and pro-invasive activity in melanoma [34,35]. Notably, the expression of TGFß2, a secreted growth factor overexpressed in several tumor types including melanomas [36], was also downregulated ~12-fold (Table S1). TGFß/SMAD pathway contributes to melanoma

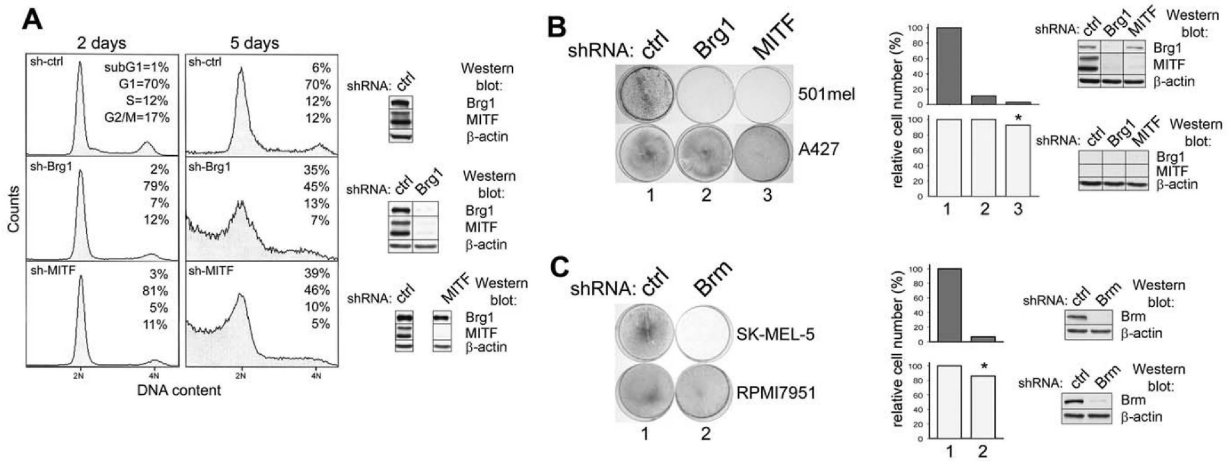

Figure 1. Flow cytometry and colony formation of melanoma cells after knockdown of BRG1, MITF or BRM. A. Effect of BRG1 or MITF depletion on DNA content profile determined by flow cytometry in 501mel cells. Cells were analyzed 2 days after selection in 2.5 μg/ml puromycin (left), or combined (detached and adherent) cells were analyzed 5 days after selection (right). All cells killed by puromycin were removed shortly after antibiotic selection and only puromycin resistant cells were assayed. Right, Western blots verifying the knockdown efficiency for BRG1 and MITF were performed after 2 days followimg the selection. **B.** BRG1 or MITF depletion inhibits colony formation in 501mel cells. A427 cells (BRG1 and BRM negative) were not affected by sh-BRG1. Middle, the quantitation of cells on dishes shown on the left. Right, Western blots verifying the knockdown efficiency for BRG1 and MITF. **C.** BRM depletion inhibits cell growth in BRG1-negative SK-MEL-5 melanoma cells, but not control RPMI7951 melanoma cells, which are BRM and BRG1-positive [26] but MITF-negative [33]. Right, Western blots showing the efficient shRNA-mediated BRM downregulation. *, not statistically significant ($P<0.01$).
doi:10.1371/journal.pone.0054110.g001

invasiveness [36,37] and TGFß2 has been shown to be an important determinant of melanoma brain metastases [38]. An antiapoptotic protein survivin was downregulated ~1.9-fold (data not shown). Of the smaller set of >8-fold upregulated genes, 10 probes (out of 23) detected collagen type XIIIα1, which was previously reported to participate in epidermal cell-cell adhesions [39]. An efficient acute suppression of BRG1 expression was achieved in cells in which microarray analysis was performed, as shown by W. blot (Figure 3B). Additionally, the cell cycle driving protein E2F1 was downregulated about ~4-fold (Table S1) and the cdk inhibitor p15 was upregulated ~5-fold (Table S3). These changes might have contributed to the antiproliferative phenotype after BRG1 depletion. Collectively, the microarrays confirmed the requirement of BRG1 for the maintenance of pigment cell-specific transcription regulated by MITF and, importantly, revealed a positive role of SWI/SNF for expression of several novel prosurvival molecules in melanoma cells, which are not known to be connected to the MITF axis.

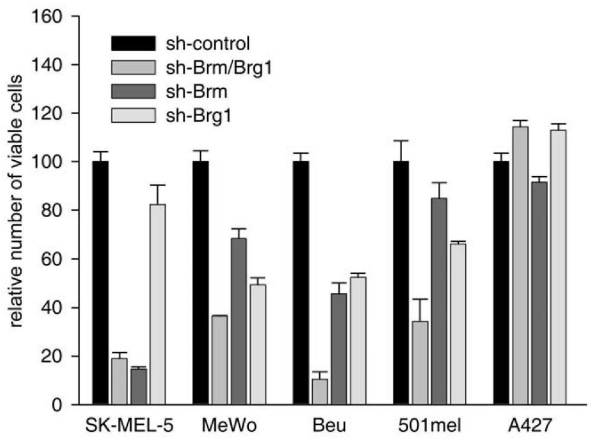

Figure 2. Viability of melanoma cell lines after shRNA-mediated depletion of BRG1, BRM, or both. Viability of each cell line is indicated in the histogram. The results are expressed as per cents of control shRNA-transduced cells. The cells were infected with appropriate lentivirus, replated two days later into 12-well plates, and viability was determined after next 3 days. The values are means of triplicates +-SE. One of the three independent experiments is shown. No decrease of viability was observed in BRM/BRG1-null A427 cells, used as a negative control. The knockdown of BRG1, BRM or common BRG1/BRM was efficient (see Figures 1, 3B, and 4, and Figure 3 in our previous article [26]).
doi:10.1371/journal.pone.0054110.g002

Verification of Decrease of Prosurvival Molecules in Melanoma Cells

Although most of the effects caused by BRG1 knockdown on proliferation could be mediated by repression of MITF and hence its targets, several prosurvival factors (OPN, IGF1, survivin and TGFß2), the transcription of which was not reported to be MITF-responsive, were downregulated by BRG1 depletion, as revealed by microarray analysis. All of these proteins were previously implicated as pro-oncogenic factors in melanoma, promoting proliferation, antiapoptosis, invasiveness, or metastasis [30,34,37,40]. To validate the microarray results, we estimated the protein expression and found that protein levels of OPN, survivin, TGFß2, and also livin, a MITF transcriptional target added as a control for MITF-driven gene, were substantially decreased in BRG1-depleted cells (Figure 4A). Sox10 (an upstream MITF regulator) was also decreased, but to a lesser extent (Figure 4A). We were not able to detect an appreciable expression level of the very small molecule IGF1 on W. blots, probably because this autocrine factor could be secreted early from cells and stimulates growth through the binding to its receptors. Nevertheless, IGF1 mRNA was lowered in 501mel cells (Figure 4B). The mRNA levels of OPN, livin, survivin, IGF1, TGFß2, and Sox10

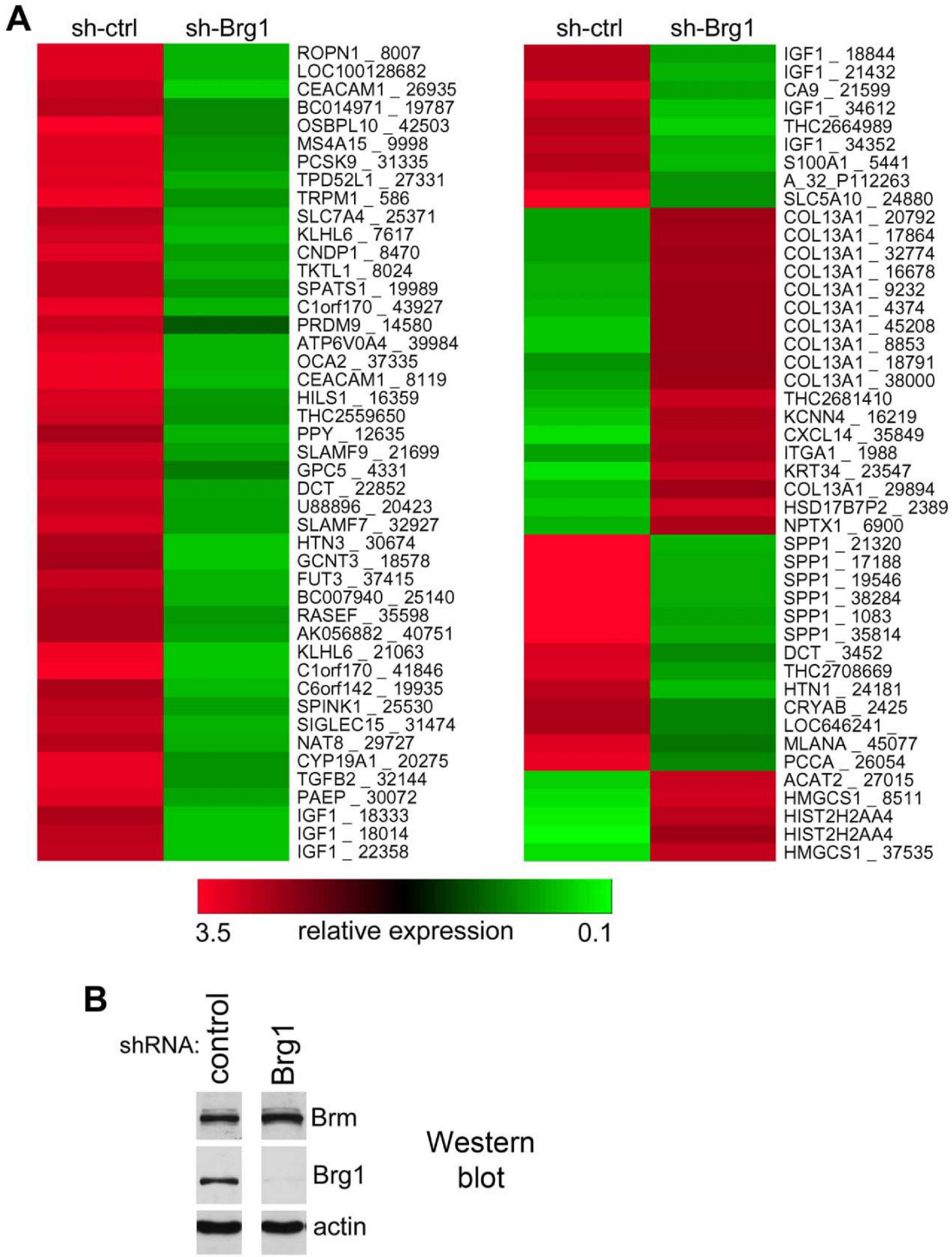

Figure 3. Microarrays showing transcriptional response of BRG1 knockdown in 501mel cells. A. Microarray analysis of genes that are differentially expressed between sh-control-transfected and sh-BRG1-transfected 501mel cells. Heat map shows only genes whose expression was changed more than 8-fold ($P<0.005$). Color key (bottom, the highest expression is in red color) shows the relative range of expression. Genes the expression of which was down- or up-regulated >2-fold ($P<0.005$) are listed in Tables S1 and S3, respectively. Control and BRG1-shRNA transfected 501mel cells were analyzed immediately after a short (2-day) selection in puromycin to prevent cell death or reexpression of MITF due to the persisting BRM [26]. Two experiments (in duplicates) were carried out with similar results and one is presented. **B.** Western blot confirming the

efficiency of BRG1 knockdown in cells which were used for microarray analysis. BRM protein expression was not appreciably affected (not shown and [26]).
doi:10.1371/journal.pone.0054110.g003

were also significantly reduced, by RT-PCR (Figure 4B) and real-time PCR (for survivin, 5-fold, data not shown). By contrast, the mRNA levels of two antiapoptotic proteins Bcl2 and Mcl-1 remained unchanged, by real time PCR (not shown). To further verify that all these protumorigenic factors were downregulated after BRG1 depletion also in different melanoma cells, we used melanoma cell line MeWo, where we proved that the same factors were downregulated similarly as in 501mel cells (Figure 4C). Thus, these factors generally need the presence of BRG1 for their expression in melanoma cells.

Expression of OPN, IGF1, TGFß2, and Survivin is not Affected by MITF Knockdown in Melanoma Cells

The expression of OPN, IGF1, TGFß2, and survivin was not reported previously to be MITF-dependent in melanomas. Nevertheless, we wished to exclude that the decrease of these molecules were caused by the depletion of MITF or genes downstream of its cascade. We blocked MITF in 501mel cells by shRNA and performed a Western blot of an extract from both blocked and control cells. As expected, none of the OPN, TGFß2, survivin, and Sox 10 proteins was affected by severe MITF downregulation, while expression of livin, a MITF target, almost disappeared (Figure 4D). Thus, we verified that the above cancer prosurvival factors and Sox10 require BRG1, not MITF, for expression.

Expression of BRG1 and BRM in Melanoma Tumor Specimens and Nevi

The results suggest that the presence of either BRG1 or BRM is pivotal for MITF expression [26]. We therefore asked whether cells in the tumor tissue maintain expression of at least one ATPase. Immunohistochemical analysis (Figure 5) revealed that BRM protein was highly expressed in nevi (5 analyzed samples) and tumors (9 samples of primary melanomas) (score predominantly 3); no BRM-negative cells were found. Detection of BRG1 generally showed more heterogeneous staining (score 2 or 3) with a higher proportion of moderate expression levels in nevi compared with melanomas. Also, increased number of weakly stained nuclei and very rare BRG1-negative cells (score 0) were detected in some melanoma specimens. MITF immunostaining showed heterogeneity of expression in nevi and melanomas (score 0–3) with some cells being devoid of MITF expression (Figure 5), in concert with previous findings [10]. The results together show that both ATPases are expressed in melanomas and whereas BRG1 expression might be attenuated or rarely entirely lost in some tumor cells, BRM positivity is maintained in nevi and primary melanomas.

Partial Rescue of Cell Proliferation by CDK2, Osteopontin, and IGF1

Next, we explored the consequences of overexpressing several prosurvival proteins and MITF on proliferation of BRG1-depleted cells. Addition of recombinant OPN protein alone rescued the colony formation by about ~2-fold (Figure 6A), whereas individually overexpressed livin, survivin, or Bcl-2 failed to show any effect (data not shown). Overexpression of the MITF-Vp16 chimera, a transcriptionally more active MITF derivative [41], only insignificantly increased the number of colonies, presumably because the expression of some downstream targets involved in proliferation are also SWI/SNF-dependent [25], while the MITF target CDK2 was more effective (Figure 6B). Furthermore, the presence of IGF1 protein in the culture medium increased proliferation of BRG1-blocked cells in a dose-dependent manner up to about 3~fold (Figure 6C). Together, although the individual

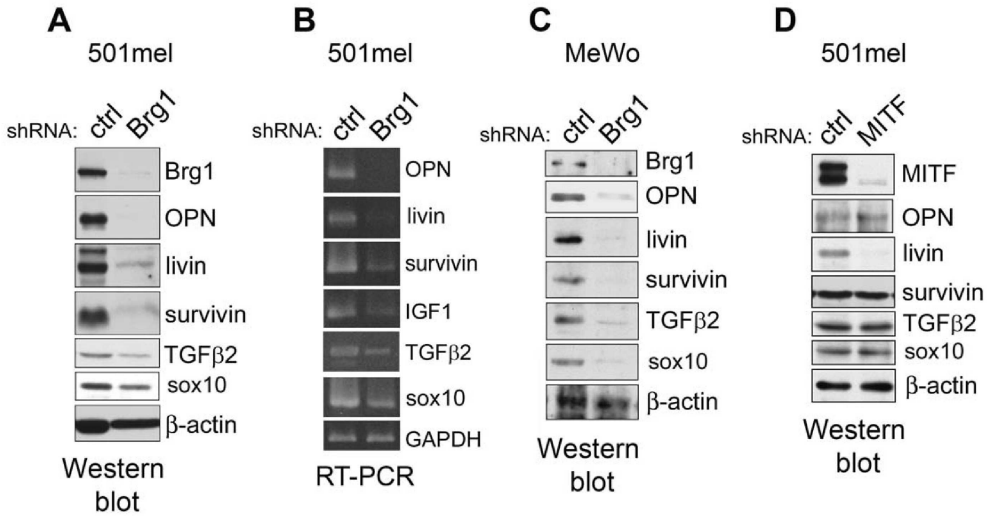

Figure 4. Several prosurvival molecules are decreased in BRG1-depleted cells and are not affected by sh-MITF. A. Decreased protein expression of OPN, livin, survivin, TGFß2 and Sox10 after BRG1 silencing in 501mel cells. **B.** Decreased mRNA levels for the same proteins after BRG1 silencing in 501mel cells. **C.** Similar decrease of expression levels for the proteins analyzed in (A) performed in MeWo cells. **D.** Verification that OPN, survivin, TGFß2 and Sox10 are not regulated by MITF since MITF depletion in 501mel cells did not have any effect on their protein levels. Livin alone was decreased because it is a MITF target. ß-actin used as a control was not changed in all experiments.
doi:10.1371/journal.pone.0054110.g004

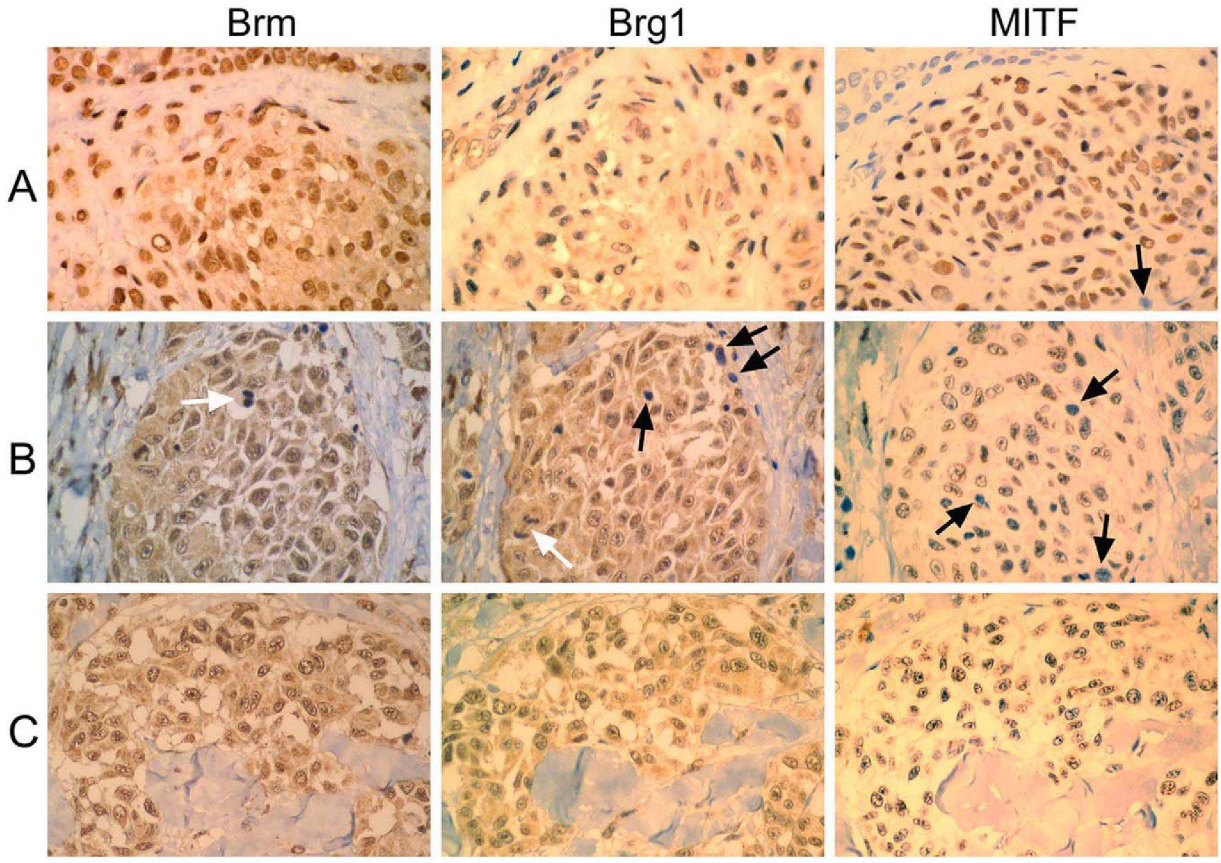

Figure 5. Immunostaining of BRM, BRG1, and MITF in nevi and primary melanomas. A. Intradermal nevus; **B** and **C.** Primary melanomas. Parallel sections (5 μm) were stained with antibodies against BRM (left), BRG1 (middle), and MITF (right). Protein expression was analyzed in intradermal or compound nevi (5 sections) and primary melanomas >1 mm in thickness (9 sections) and representative images were shown. Black arrows indicate negative interphase nuclei in BRG1 and MITF staining, open arrows mark BRM- or BRG1-negative mitotic nuclei. Scoring (0–3) is described in Materials and Methods. Magnification, ×400.
doi:10.1371/journal.pone.0054110.g005

effects of exogenously supplemented molecules on restoring growth were generally mild, the results suggest that mainly the decreased CDK2 and OPN levels may be partly responsible for the proliferation defect and a combined decrease of survival factors might contribute to growth inhibition and apoptosis, either directly or indirectly, elicited by blocking the SWI/SNF function in melanoma cells.

Discussion

Although both BRG1 and BRM display extensive homology and are interchangeable in function to support MITF and its targets expression in melanoma, their function in SWI/SNF complexes may not be equivalent. For example, these ATPases were differentially required for the expression of p53-induced p21 and MDM2 [42]. Also, an opposite role of BRM- versus BRG1-containing complexes was suggested during osteoblast differentiation [43] and maintenance of chromatin integrity was specifically dependent on BRG1 [44]. Our previous results suggest that BRG1 is fully capable of maintaining MITF expression and its acute knockdown abrogate several MITF target genes. Nevertheless, BRM can also support MITF expression in 501mel cells if it is present as the only ATPase in melanoma cells [26]. A similar compensatory role of BRM has been described previously when BRM was able to restore the Rb-dependent sensitivity to cell cycle arrest [45] or coactivate the induction of p21 in BRG1-knocked down MCF7 cells [42].

Several reports have suggested that BRM may constitute an important constraint against malignant transformation. In normal melanocytes, BRM is transiently recruited into Rb- and HDAC1-containing complexes when heterochromatin has been formed during cellular senescence [46]. Loss of BRM also promoted proliferation of prostate epithelial cells [47] and was associated with poorly differentiated gastric carcinomas [48]. Expression of BRM was also found silenced in cell lines derived from multiple tumor types through an epigenetic mechanism and restoration of its expression by HDAC (histone deacetylases) inhibitors was considered as an approach in cancer therapy [17]. By contrast, we observed that BRM was expressed not only in all melanoma cell lines analyzed, but also in nevi and primary melanomas.

Becker et al. [49] recently described undetectable BRG1 protein in more than a half of primary and metastatic melanomas while BRM negativity was much less frequent. Although BRG1 negativity in their work was much more frequent than detected here, the tendency of preferential BRG1 loss with retained BRM expression was similar in both studies. Thus, BRM might become

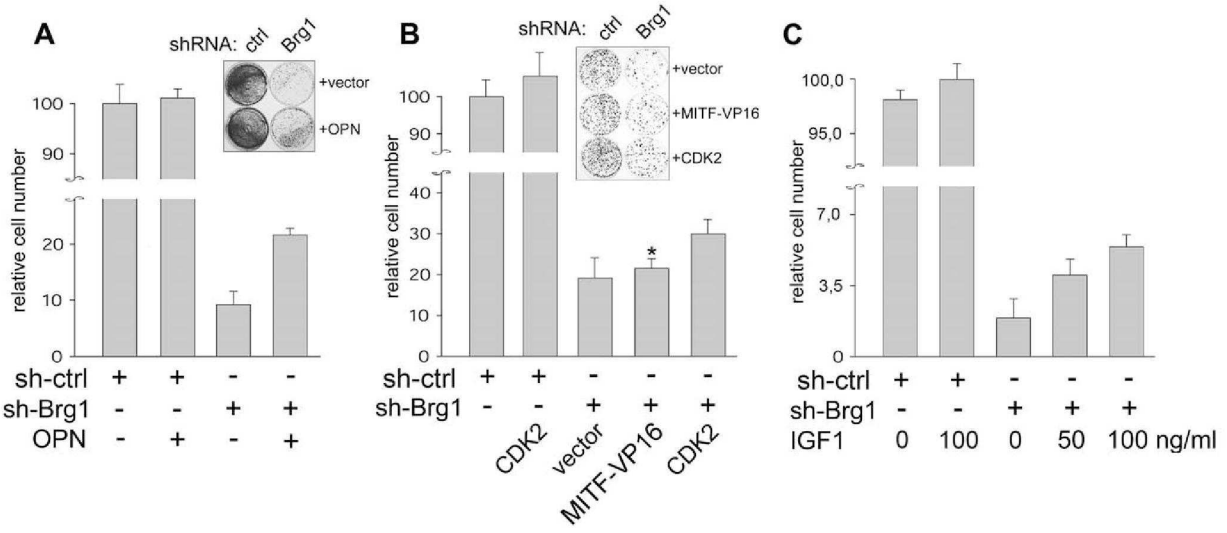

Figure 6. Separate restitution of OPN, CDK2 or IGF1 partly rescued cell proliferation. A. Addition of recombinant OPN (50 μg/ml) can partly increase colony formation of BRG1-silenced 501mel cells. OPN has been added 1 day after shRNA encoding plasmid and fresh OPN was replaced two days later. Cells were analyzed 5 days after transfection. The values are means of duplicates +-SE. One of four independent experiments is shown. **B.** Overexpression of CDK2 (by use of a CDK2-FLAG expression plasmid), but not the MITF-VP16 chimera [41] slightly rescues colony formation of BRG1-silenced cells 501mel cells. *, not statistically significant ($P<0.05$), compared to control. CDK2-FLAG was efficiently expressed, estimated by Western blot (not shown). **C.** Exogenously added recombinant IGF1 protein partly restores colony formation of BRG1-depleted 501mel cells in a dose-dependent manner. No effects of added recombinant factors or CDK plasmid were seen in control shRNA cells. The values are means of duplicates +-SE. Western blot detecting OPN and RT-PCR detecting IGF1 mRNA showed no increase of their levels in cell extract (not shown), evidently because these factors are added as recombinant proteins and exert their effects via cell receptors. In B and C, one of three independent experiments is depicted. Relative cell number was estimated by staining with crystal violet as described in Materials and Methods.
doi:10.1371/journal.pone.0054110.g006

a crucial remaining ATPase maintaining the expression of MITF and perhaps additional new targets described here in melanoma cells in vivo under conditions of BRG1 loss [26], especially in later stages of tumor progression. In tumor specimens, we also observed MITF-negative cells, which were evidently BRM/BRG1-positive. It is possible that these cells survive and proliferate due to the expression of multiple pro-proliferative and antiapoptotic non-MITF-targets identified here.

Our recent silencing experiments further indicate that melanoma cells exert defects in proliferation when both BRG1 and BRM are depleted [26], which could be attributed to the loss of MITF expression. However, reexpression of hyperactive MITF-Vp16 construct is not sufficient to restore growth (Figure 6B), presumably because the expression of proliferation-related downstream targets are also SWI/SNF-dependent [25] and/or other factors besides MITF, as described here, may be important for survival. Moreover, since melanoma lines were found to be differentially sensitive to MITF depletion [50], MITF target genes might play distinct roles in survival of individual cell lines. We also observed prominent increase of collagen type XIII expression in BRG1-blocked cells, and a similar induction of this molecule involved in cell-cell contact has been described earlier in cancer cells, though stromal ones [51], and proposed to have pro-metastatic properties.

Recently, other authors described the appearance of senescent cells rather than apoptosis upon inhibition of MITF expression [52,53]. These results are not so contradictory and could be explained by different experimental design employed by Giuliano et al. [53], who used RNA duplexes for MITF inhibition, a short pulse of transfection, and used conditioned medium in some experiments [52]. Instead, we used a plasmid producing continuously very efficient sh-BRG1. In our experiments, several days after transfection, the cells were grossly deteriorated with a large portion of detached cells and apoptosis was detected. According to the postulated rheostat model for MITF levels in melanoma cells [10], small changes of MITF level can cause very different cellular effects. We argue that in the described experiments [53] MITF could have dropped to a low level causing senescence, while sustained complete inhibition of MITF expression may indeed lead to apoptosis in 501mel cells.

Importantly, our results further indicate that the SWI/SNF complex may be crucial for the activity of MITF-independent pathways resulting in upregulation of critical factors such as OPN or IGF1. Both of these molecules were capable of rescuing proliferation of BRG1-depleted 501mel cells, albeit their overall effect was mild. Presumably, combined silencing of MITF and prosurvival growth factors participated in loss of proliferation in BRG1-depleted cells. The expression of a secreted phosphoprotein OPN, which is involved in tumor progression and metastasis, is increased in many kinds of tumors including melanomas [34] and OPN promoter was shown to be a target of activated Hedgehog pathway in melanoma [40]. The Hedgehog-GLI signaling is activated in melanoma and interference with this pathway inhibits melanoma growth and prevents recurrence and metastases in mice [54]. It remains to be investigated if SWI/SNF could activate transcriptional responses to aberrantly activated Hedgehog pathway, and whether BRG1 is involved directly in transcription from promoters of IGF1, OPN, survivin, or TGFß2. Recent search for driver and passenger mutations in melanoma on the whole-genome basis by the use of exome sequencing revealed abundant passenger mutations caused evidently by UV light exposure. Only one Brg1 nonsense mutation and no Brm mutations were found [55]. These data indicate that the Brg1 and Brm are not a subject of driver and passenger mutations and indirectly support our current results that SWI/SNF ATPases are

preserved intact in melanoma cells and the SWI/SNF complex plays a prosurvival role in this cancer type. Mutations of ARID2 (BAF200), ARID1A (BAF250A) and ARID1B (BAF250B), which are also SWI/SNF components [14], were also found mutated at relatively low frequencies and ARID2 mutations were predicted to be loss of function events. However, no data about the function of ARID proteins and the functional consequence of their mutations in melanoma cells are available. Evidently, more investigation is needed to delineate the function of ARIDs in melanoma in the context of SWI/SNF. In summary, as BRG1 knockdown downregulated expression of a large number of MITF-dependent and MITF-independent prosurvival proteins, our data suggest that a tissue-aimed inactivation of the SWI/SNF complex might become an effective approach in the therapy of melanoma.

Materials and Methods

Ethics Statement

The samples of patients (primary melanoma lesions and benign nevi) used in this work were obtained with the patients written informed consent. The Ethics Committee of the University Hospital Bulovka, Prague, approved the written informed consent and agreed with the analysis of samples which was performed in the present work.

Cell Lines

501mel cells were gift from Dr. R. Halaban (Yale University, New Haven, CT) and were previously published [10,11,52,53]. 501mel cells were maintained in the RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 293FT cells (Life Technologies, Carlsbad, CA) were maintained in the DMEM medium supplemented with FBS. A427 lung carcinoma cells (ATCC, Manassas, VA) were grown in the RPMI-1640 medium and SK-MEL-5 and MeWo melanoma cells (ATCC) were maintained in the E-MEM medium supplemented with FBS, pyruvate, and non-essential amino acids (Sigma). Beu melanoma cells were described [33]. All cell lines contained 1% penicillin and 1% streptomycin in the medium.

Plasmids, Transfections, and Chemicals

CDK2 cDNA was expressed from the pFLAG-CMV-4 (Sigma, St. Louis, MO). The MITF-Vp16 construct was described [41]. Cells were transfected using Lipofectamine 2000 (Life Technologies) and selected in 2.0 μg/ml puromycin for indicated time intervals. Recombinant human osteopontin and IGF1 were purchased from Sigma. Other chemicals were from Sigma.

shRNA Knockdowns and Lentiviruses

The shRNA sequences were cloned in the pSUPER.puro vector (Oligoengine, Seattle, WA). The target sequence for knockdown of human MITF was GGACAATCACAACCTGATTG. The BRM, BRG1, and the common BRM/BRG1 target sequences were described previously [56], and are as follows: BRM, GGAGGTGCTAAGACACTTATG; BRG1, GTAGCTCC-GAGGTCTGATAG; common BRM/BRG1, GCTGGAGAAG-CAGCAGAAG. The non-targeting sequence was GACGG-TACCGCGAACGAGC. Generation of lentiviral vectors (by using pLVTHM plasmid) (Addgene, Cambridge, MA) and lentiviruses were performed as described [26]. We used virus titers that infected nearly 100 per cent of cells (as assessed by GFP positivity). Cells were infected in the presence of 8 μg/ml Polybrene for 48 hrs, harvested or replated for analyses after the time intervals as indicated.

Immunoblotting and Immunohistochemistry

Whole cell extracts were subject to Western blot analysis. Anti-MITF antibody (D5) was from Labvision (Kalamazoo, MI), and anti-ß-actin from Sigma. Other antibodies used were from Santa Cruz Biotechnology (Santa Cruz, CA) unless otherwise indicated. Primary antibodies used were against BRG1 (G-7), osteopontin (LFMb-14), livin/ML-IAP (H-90), IGF1 (H-70), and survivin (D-8). Anti-Sox10 antibody was from Abcam (Cambridge, UK) (ab25978). Proteins were visualized using ECL (GE Life Sciences, Buckinghamshire, UK) according to the manufacturer's instructions.

Primary melanoma lesions and benign nevi were obtained with the patients informed consent and with the approval of the Ethics Committee of the University Hospital (see Ethics statement above). Parallel tissue sections were stained with primary antibodies against BRM (Abcam, ab15597), BRG1 (H-88, Santa Cruz) or MITF (D5, Dako, Glostrup, Denmark). The detection of antigen/antibody complexes were performed using EnVision+ avidin-biotin detection system (Dako). Because Brm and Brg1 contain high sequence similarity, the specificity of primary antibodies were verified by Western blot and immunofluorescence in Brg1+/Brm- (A549) and Brg1−/Brm+ (G401) cell lines (not shown) and antibodies were proved to be strictly specific. Score was obtained for each sample of tissue section. Sections were independently examined by two pathologists. Tissues were scored on a scale of 0 (negative) to 3 (highly positive) based on the intensity of staining.

Reverse Transcription-PCR

Total RNA was extracted from cells using TRIZOL reagent (Life Technologies) and cDNA was synthesized from 2 μg of total RNA using the SuperScript II RNase H- reverse transcriptase (Life Technologies) with an oligo-dT primer. Detection of gene expression by RT-PCR was carried out with primers pairs purchased from Santa Cruz (for OPN, livin, survivin, IGF1, TGFß2, and Sox10). Primers for the detection of control GAPDH gene expression were 5′-TGAAGGTCGGAGTCAACG-GATTTGGT (fw) and 5′-CATGTGGGCCATGAGGTCCAC-CAC (rev).

Apoptosis, Colony Formation, and Viability Assays

Cells were evaluated for DNA content by flow cytometry. Cells were washed, incubated in 0.2mol/l sodium phosphate-0.1mol/l citric acid solution (pH 7.8), digested with RNase A and stained with propidium iodide before analysis in a FACSCalibur (BD Biosciences, San Jose, CA). Results were analyzed by CellQuest software. Detection of apoptosis with TUNEL assay was performed by the use of APO-DIRECT^{TM} kit (BD Biosciences) according to manufacturers instructions without the propidium iodide staining. Microscopic images of apoptotic nuclei in cultured cells were taken on IX51 Olympus fluorescence microscope. Caspase 3/7 activities were detected with a kit obtained from Enzo Life Sciences (Farmingdale, NY). For colony formation assays, cells were replated two days after transfection into the puromycin-containing medium, and stained with crystal violet after 12 days. Cell viability was assessed by growing lentivirus-infected cells in 24-well plates and staining with the MTT cytotoxicity/proliferation kit (Sigma) in triplicate samples. No decrease of viability was observed in BRM/BRG1-null A427 lung carcinoma cells, which were used as a negative control (Figure 2). For cell growth quantitation, cells were fixed and stained with crystal violet and the stain was then dissolved in wells with acetic acid/ethanol solution and the relative number of cells was estimated by spectrophotometry.

Microarrays

Agilent Whole Human Genome (4x44K) expression arrays (Agilent Technologies, Santa Clara, CA) were used according to protocols recommended by the manufacturer. Total RNA was isolated using TRIZOL reagent. RNA labeling, hybridization, scanning and data acquisition was performed at AtlasBiolabs (Berlin, Germany). Primary data were generated with Agilents Feature Extraction Software (version 10.5.1.1) from which differential gene expression ratio values were calculated. Data analysis was done using the R-project (v. 2.8.1, www.r-project.org) and BioConductor (v. 2.3, www.bioconductor.org) software. Adjusted P value was calculated by the method of Benjamini-Hochberg.

Statistical Methods

Statistical analyses of proliferation rates, real-time RT-PCR, and luciferase assays were done using Student's t test.

Supporting Information

Figure S1 Apoptotic nuclei in sh-MITF and sh-BRG1 treated cells. Apoptosis was detected by DAPI staining of nuclear DNA. Mounting of the remaining attached cells was performed in Vectashield mounting medium with DAPI five days after puromycin selection and observed on Olympus IX51 Olympus fluorescence microscope. Nuclei with irregular and lobular DNA staining, condensed DNA, and fragmented DNA were scored on 100 independent cells and 9 and 11 apoptotic nuclear patterns were seen in sh-MITF and sh-BRG1 cells, respectively, while no nuclei of sh-control cells showed apoptotic signs. Two representative images are shown for each shRNA (left and right panels).
(TIF)

Figure S2 Flow cytometry of detached and adherent cells. We performed separate flow cytometry profiles after 5 days after a 2-day puromycin selection following transfection of appropriate shRNA, similarly as in Figure 1. High content of sub-G1 and G1 phase of the cell cycle was visible in floating cell profiles. Remaining adherent cells showed normal-like profile of DNA content. Absence of G2/M phase in floating cells indicates that these cells ceased proliferating.
(TIF)

Figure S3 TUNEL assay for the detection of apoptosis performed on 501mel cells. Flow cytometric measurements for sh-control, sh-BRG1 and sh-MITF transfected cells after 5 days of selection in puromycin. Two days after transfection, the medium was changed to remove puromycin-killed cells. No cells remained after puromycin selection in the sample where no plasmid was present. Pooled adherent and detached cells were analyzed. No detached cells were seen in the sh-control transfected cells. The second peak indicates the extent of apoptosis, and this was more prominent in sh-MITF cells (42%) than in sh-BRG1 cells (18%) (see Results for explanation). Apoptosis was negligible (3%) in sh-control.
(TIF)

Table S1 Microarray analysis of genes down-regulated >2-fold (P<0.005) by BRG1 depletion in 501mel cells.
(PDF)

Table S2 Known MITF target genes downregulated more than 2-fold by BRG1 knockdown in 501mel cells (P<0.005). (MITF itself was downregulated ~5-fold).
(PDF)

Table S3 Microarray analysis of genes up-regulated >2-fold (P<0.005) by BRG1 depletion in 501mel cells.
(PDF)

Acknowledgments

We thank V. Stránecký for analysis of microarray data and Č. Vlček for DNA sequencing. We also thank R. Halaban for providing the 501mel cells. In addition we thank V. Šmíd for help with microscopic images of apoptotic cells. L.O. participates in the Ph.D. program at Charles University in Prague, Third Faculty of Medicine, Czech Rep. We appreciate a general support from the First Faculty of Medicine, Charles University in Prague.

Author Contributions

Conceived and designed the experiments: LO JV. Performed the experiments: LO JV JR PŽ KB. Analyzed the data: LO JV KB. Contributed reagents/materials/analysis tools: LO JV KB. Wrote the paper: JV.

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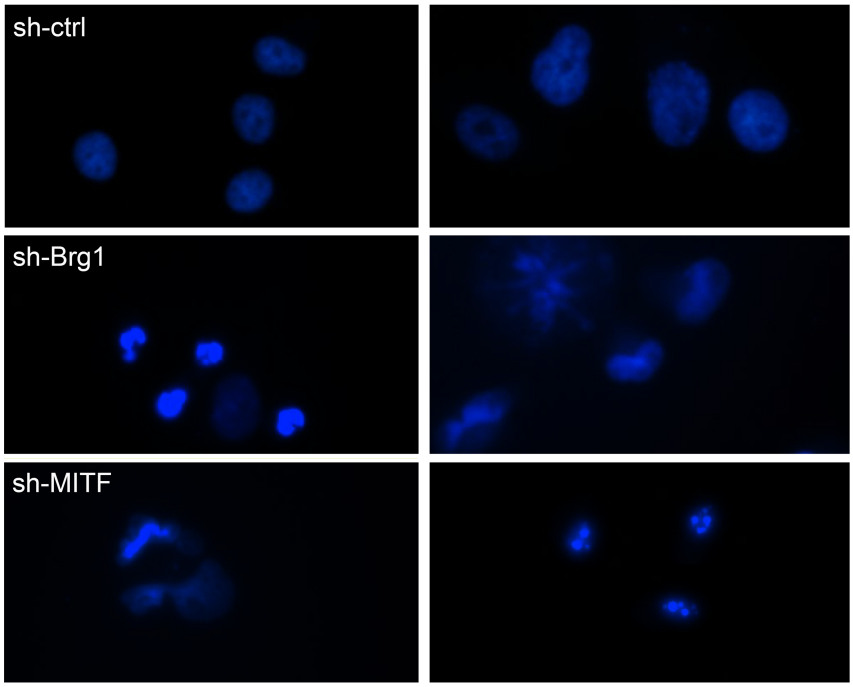


Figure S1

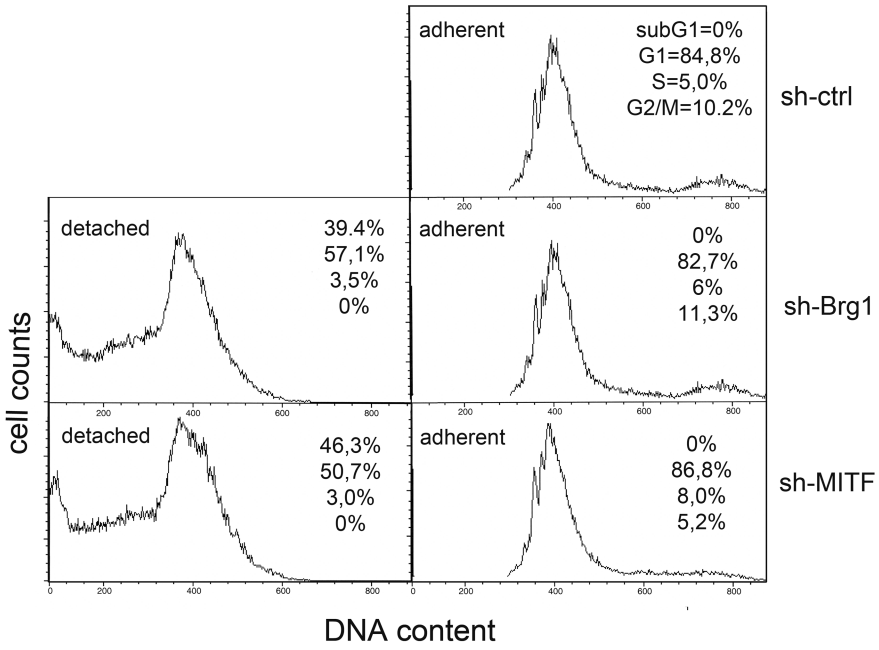
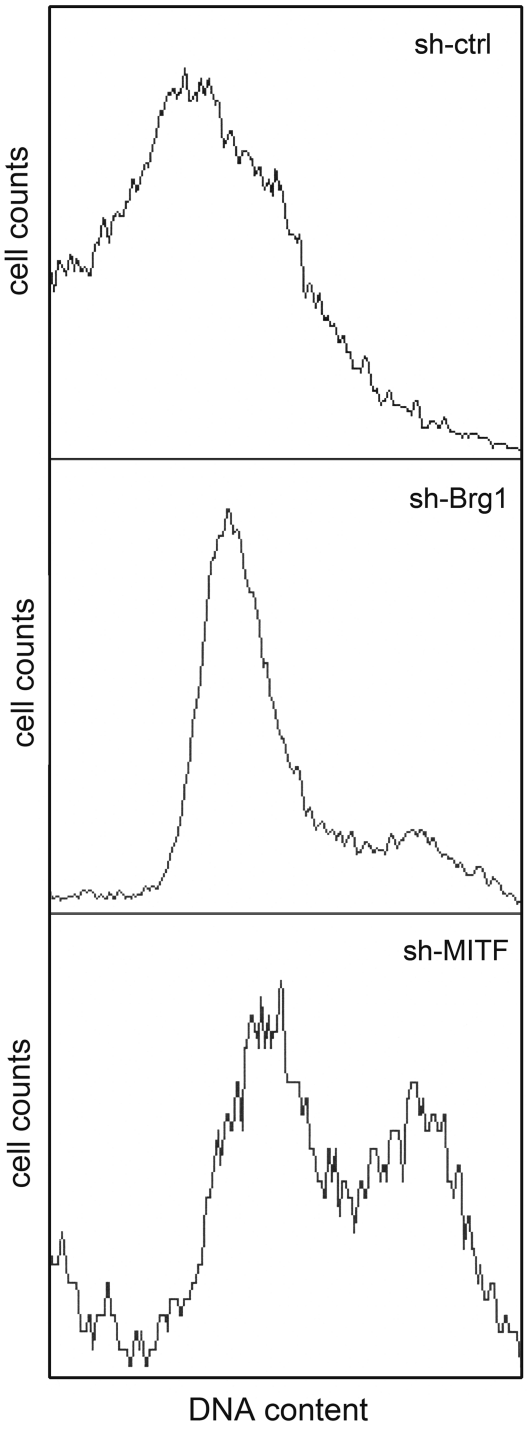


Figure S2


sh-ctrl
cell counts
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DNA content

Figure S3

Supplementary Table S1. Microarray analysis of genes down-regulated >2-fold (P<0.005) by Brg1 depletion in 501mel cells.

Gene Symbol	Systematic Name	Gene Title	Fold change
SLAMF7	NM_021181	ref|Homo sapiens SLAM family member 7 (SLAMF7), mRNA [NM_021181]	34,23
HTN1	NM_002159	ref|Homo sapiens histatin 1 (HTN1), mRNA [NM_002159]	23,30
DCT	NM_001922	ref|Homo sapiens dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2) (DCT), transcript variant 1, mRNA [NM_001922]	22,50
U88896	U88896	gb|Human endogenous retrovirus H protease/integrase-derived ORF1, ORF2, and putative envelope protein mRNA, complete cds. [U88896]	20,49
DCT	NM_001922	ref|Homo sapiens dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2) (DCT), transcript variant 1, mRNA [NM_001922]	19,45
GPC5	NM_004466	ref|Homo sapiens glypican 5 (GPC5), mRNA [NM_004466]	19,44
PAEP	NM_002571	ref|Homo sapiens progestagen-associated endometrial protein (PAEP), transcript variant 2, mRNA [NM_002571]	18,93
PCSK9	NM_174936	ref|Homo sapiens proprotein convertase subtilisin/kexin type 9 (PCSK9), mRNA [NM_174936]	18,43
PPY	NM_002722	ref|Homo sapiens pancreatic polypeptide (PPY), mRNA [NM_002722]	17,89
MS4A15	NM_152717	ref|Homo sapiens membrane-spanning 4-domains, subfamily A, member 15 (MS4A15), transcript variant 2, mRNA [NM_152717]	17,39
TPD52L1	NM_001003395	ref|Homo sapiens tumor protein D52-like 1 (TPD52L1), transcript variant 2, mRNA [NM_001003395]	16,78
TRPM1	NM_002420	ref|Homo sapiens transient receptor potential cation channel, subfamily M, member 1 (TRPM1), mRNA [NM_002420]	15,98
SLAMF9	NM_033438	ref|Homo sapiens SLAM family member 9 (SLAMF9), mRNA [NM_033438]	15,86
THC2708669	THC2708669	thc|Q3IE62_PSEHT (Q3IE62) Dihydropteroate synthase (DHPS) (Dihydropteroate pyrophosphorylase) , partial (5%) [THC2708669]	13,99
SLC5A10	NM_152351	ref|Homo sapiens solute carrier family 5 (sodium/glucose cotransporter), member 10 (SLC5A10), transcript variant 1, mRNA [NM_152351]	13,48
CEACAM1	NM_001712	ref|Homo sapiens carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein) (CEACAM1), transcript variant 1, mRNA [NM_001712]	13,14
ROPN1	NM_017578	ref|Homo sapiens ropporin, rhophilin associated protein 1 (ROPN1), mRNA [NM_017578]	12,09
TGFB2	NM_001135599	ref|Homo sapiens transforming growth factor, beta 2 (TGFB2), transcript variant 1, mRNA [NM_001135599]	12,04
TKTL1	NM_012253	ref|Homo sapiens transketolase-like 1 (TKTL1), mRNA [NM_012253]	11,97
LOC100128682	AK128099	gb|Homo sapiens cDNA FLJ46220 fis, clone TESTI4013774. [AK128099]	11,43
PRDM9	NM_020227	ref|Homo sapiens PR domain containing 9 (PRDM9), mRNA [NM_020227]	11,26
CEACAM1	NM_001024912	ref|Homo sapiens carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein) (CEACAM1), transcript variant 2, mRNA [NM_001024912]	11,26
FUT3	NM_000149	ref|Homo sapiens fucosyltransferase 3 (galactoside 3(4)-L-fucosyltransferase, Lewis blood group) (FUT3), transcript variant 1, mRNA [NM_000149]	11,12
BC007940	BC007940	gb|Homo sapiens cDNA clone IMAGE:4131853, **** WARNING: chimeric clone ****. [BC007940]	11,03
CRYAB	NM_001885	ref|Homo sapiens crystallin, alpha B (CRYAB), mRNA [NM_001885]	10,80
MLANA	NM_005511	ref|Homo sapiens melan-A (MLANA), mRNA [NM_005511]	10,76
PCCA	NM_000282	ref|Homo sapiens propionyl Coenzyme A carboxylase, alpha polypeptide (PCCA), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA [NM_	10,69
LOC646241	BC028204	gb|Homo sapiens cDNA clone IMAGE:5201079. [BC028204]	10,41
C1orf170	AK123855	gb|Homo sapiens cDNA FLJ41861 fis, clone NTONG2008672. [AK123855]	10,37
CNDP1	NM_032649	ref|Homo sapiens camosine dipeptidase 1 (metallopeptidase M20 family) (CNDP1), mRNA [NM_032649]	10,28
SPINK1	NM_003122	ref|Homo sapiens serine peptidase inhibitor, Kazal type 1 (SPINK1), mRNA [NM_003122]	10,19
SLC7A4	NM_004173	ref|Homo sapiens solute carrier family 7 (cationic amino acid transporter, y+ system), member 4 (SLC7A4), mRNA [NM_004173]	10,17
GCNT3	ENST00000267857	ens|Beta-1,3-galactosyl-O-glycosyl-glycoprotein beta-1,6-N-acetylglucosaminyltransferase 3 (EC 2.4.1.102)(EC 2.4.1.150)(Core 2/core 4 beta-1,6-N-acetylglucos	9,93
ATP6V0A4	NM_020632	ref|Homo sapiens ATPase, H+ transporting, lysosomal V0 subunit a4 (ATP6V0A4), transcript variant 1, mRNA [NM_020632]	9,91
C1orf170	AK123855	gb|Homo sapiens cDNA FLJ41861 fis, clone NTONG2008672. [AK123855]	9,77
C6orf142	NM_138569	ref|Homo sapiens chromosome 6 open reading frame 142 (C6orf142), mRNA [NM_138569]	9,71
S100A1	NM_006271	ref|Homo sapiens S100 calcium binding protein A1 (S100A1), mRNA [NM_006271]	9,65
HILS1	NR_024193	ref|Homo sapiens histone linker H1 domain, spermatid-specific 1 (HILS1), transcript variant 1, non-coding RNA [NR_024193]	9,62
SPATS1	NM_145026	ref|Homo sapiens spermatogenesis associated, serine-rich 1 (SPATS1), mRNA [NM_145026]	9,47
IGF1	NM_000618	ref|Homo sapiens insulin-like growth factor 1 (somatomedin C) (IGF1), transcript variant 4, mRNA [NM_000618]	9,41
CYP19A1	NM_031226	ref|Homo sapiens cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1), transcript variant 2, mRNA [NM_031226]	9,29
IGF1	NM_000618	ref|Homo sapiens insulin-like growth factor 1 (somatomedin C) (IGF1), transcript variant 4, mRNA [NM_000618]	9,23
OSBPL10	NM_017784	ref|Homo sapiens oxysterol binding protein-like 10 (OSBPL10), mRNA [NM_017784]	9,19
IGF1	NM_000618	ref|Homo sapiens insulin-like growth factor 1 (somatomedin C) (IGF1), transcript variant 4, mRNA [NM_000618]	9,17
RASEF	NM_152573	ref|Homo sapiens RAS and EF-hand domain containing (RASEF), mRNA [NM_152573]	8,96
BC014971	BC014971	gb|Homo sapiens, Similar to tubulin, beta, 2, clone IMAGE:4873024, mRNA. [BC014971]	8,94
CA9	NM_001216	ref|Homo sapiens carbonic anhydrase IX (CA9), mRNA [NM_001216]	8,84
SPP1	NM_001040058	ref|Homo sapiens secreted phosphoprotein 1 (SPP1), transcript variant 1, mRNA [NM_001040058]	8,54
KLHL6	NM_130446	ref|Homo sapiens kelch-like 6 (Drosophila) (KLHL6), mRNA [NM_130446]	8,51
OCA2	NM_000275	ref|Homo sapiens oculocutaneous albinism II (OCA2), mRNA [NM_000275]	8,43
SIGLEC15	NM_213602	ref|Homo sapiens sialic acid binding Ig-like lectin 15 (SIGLEC15), mRNA [NM_213602]	8,41
IGF1	NM_000618	ref|Homo sapiens insulin-like growth factor 1 (somatomedin C) (IGF1), transcript variant 4, mRNA [NM_000618]	8,38
IGF1	NM_000618	ref|Homo sapiens insulin-like growth factor 1 (somatomedin C) (IGF1), transcript variant 4, mRNA [NM_000618]	8,35
KLHL6	NM_130446	ref|Homo sapiens kelch-like 6 (Drosophila) (KLHL6), mRNA [NM_130446]	8,34
SPP1	NM_001040058	ref|Homo sapiens secreted phosphoprotein 1 (SPP1), transcript variant 1, mRNA [NM_001040058]	8,32
THC2664989	THC2664989	thc|Q40J89_EHRCH (Q40J89) Cation efflux protein, partial (6%) [THC2733597]	8,32
SPP1	NM_001040058	ref|Homo sapiens secreted phosphoprotein 1 (SPP1), transcript variant 1, mRNA [NM_001040058]	8,26
AK056882	AK056882	gb|Homo sapiens cDNA FLJ32320 fis, clone PROST2003537. [AK056882]	8,25
IGF1	NM_000618	ref|Homo sapiens insulin-like growth factor 1 (somatomedin C) (IGF1), transcript variant 4, mRNA [NM_000618]	8,21
THC2559650	THC2559650	thc|ALU5_HUMAN (P39192) Alu subfamily SC sequence contamination warning entry, partial (11%) [THC2559650]	8,15
SPP1	NM_001040058	ref|Homo sapiens secreted phosphoprotein 1 (SPP1), transcript variant 1, mRNA [NM_001040058]	8,14
SPP1	NM_001040058	ref|Homo sapiens secreted phosphoprotein 1 (SPP1), transcript variant 1, mRNA [NM_001040058]	8,11
NAT8	NM_003960	ref|Homo sapiens N-acetyltransferase 8 (GCN5-related, putative) (NAT8), mRNA [NM_003960]	8,08
SPP1	NM_001040058	ref|Homo sapiens secreted phosphoprotein 1 (SPP1), transcript variant 1, mRNA [NM_001040058]	8,08
IGF1	NM_000618	ref|Homo sapiens insulin-like growth factor 1 (somatomedin C) (IGF1), transcript variant 4, mRNA [NM_000618]	8,04
HTN3	NM_000200	ref|Homo sapiens histatin 3 (HTN3), mRNA [NM_000200]	8,03
A_32_P112263	A_32_P112263	Unknown	8,01
ROPN1B	NM_001012337	ref|Homo sapiens ropporin, rhophilin associated protein 1B (ROPN1B), mRNA [NM_001012337]	7,97
SPP1	NM_001040058	ref|Homo sapiens secreted phosphoprotein 1 (SPP1), transcript variant 1, mRNA [NM_001040058]	7,96
RP4-692D3.1	NM_001080850	ref|Homo sapiens hypothetical protein LOC728621 (LOC728621), mRNA [NM_001080850]	7,93
SPP1	NM_001040058	ref|Homo sapiens secreted phosphoprotein 1 (SPP1), transcript variant 1, mRNA [NM_001040058]	7,92
SCGB1D1	NM_006552	ref|Homo sapiens secretoglobin, family 1D, member 1 (SCGB1D1), mRNA [NM_006552]	7,89
TM4SF18	NM_138786	ref|Homo sapiens transmembrane 4 L six family member 18 (TM4SF18), mRNA [NM_138786]	7,88
SPP1	NM_001040058	ref|Homo sapiens secreted phosphoprotein 1 (SPP1), transcript variant 1, mRNA [NM_001040058]	7,85
GML	NM_002066	ref|Homo sapiens glycosylphosphatidylinositol anchored molecule like protein (GML), mRNA [NM_002066]	7,83
SPP1	NM_001040058	ref|Homo sapiens secreted phosphoprotein 1 (SPP1), transcript variant 1, mRNA [NM_001040058]	7,83
IGF1	NM_000618	ref|Homo sapiens insulin-like growth factor 1 (somatomedin C) (IGF1), transcript variant 4, mRNA [NM_000618]	7,83
ZNF585A	BC026081	gb|Homo sapiens zinc finger protein 585A, mRNA (cDNA clone IMAGE:4837344). [BC026081]	7,81
DENND2D	NM_024901	ref|Homo sapiens DENN/MADD domain containing 2D (DENND2D), mRNA [NM_024901]	7,78
BCAN	NM_021948	ref|Homo sapiens brevican (BCAN), transcript variant 1, mRNA [NM_021948]	7,71
TMPRSS3	NM_032405	ref|Homo sapiens transmembrane protease, serine 3 (TMPRSS3), transcript variant D, mRNA [NM_032405]	7,64
TSPAN10	NM_031945	ref|Homo sapiens tetraspanin 10 (TSPAN10), mRNA [NM_031945]	7,57
DNAJA4	NM_018602	ref|Homo sapiens DnaJ (Hsp40) homolog, subfamily A, member 4 (DNAJA4), transcript variant 1, mRNA [NM_018602]	7,52
IGF1	NM_000618	ref|Homo sapiens insulin-like growth factor 1 (somatomedin C) (IGF1), transcript variant 4, mRNA [NM_000618]	7,45
ELSPBP1	NM_022142	ref|Homo sapiens epididymal sperm binding protein 1 (ELSPBP1), mRNA [NM_022142]	7,45
CLDN14	NM_144492	ref|Homo sapiens claudin 14 (CLDN14), transcript variant 1, mRNA [NM_144492]	7,42
C8orf75	BC014230	gb|Homo sapiens cDNA clone IMAGE:4544942, partial cds. [BC014230]	7,27
THC2711870	THC2711870	thc|O60448_HUMAN (O60448) Neuronal thread protein AD7c-NTP, partial (5%) [THC2711870]	7,26
IGF1	NM_000618	ref|Homo sapiens insulin-like growth factor 1 (somatomedin C) (IGF1), transcript variant 4, mRNA [NM_000618]	7,25
SEMA6A	NM_020796	ref|Homo sapiens sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A (SEMA6A), mRNA [NM_020796]	7,14
ENST00000272035	ENST00000272035	ens|Tubulin beta-8 chain [Source:UniProtKB/Swiss-Prot;Acc:Q3ZCM7] [ENST00000272035]	7,11
RLBP1	NM_000326	ref|Homo sapiens retinaldehyde binding protein 1 (RLBP1), mRNA [NM_000326]	6,99
CRYM	NM_001888	ref|Homo sapiens crystallin, mu (CRYM), transcript variant 1, mRNA [NM_001888]	6,88
KCNAB2	NM_003636	ref|Homo sapiens potassium voltage-gated channel, shaker-related subfamily, beta member 2 (KCNAB2), transcript variant 1, mRNA [NM_003636]	6,87
CDH7	NM_004361	ref|Homo sapiens cadherin 7, type 2 (CDH7), transcript variant b, mRNA [NM_004361]	6,86
CAMK1G	NM_020439	ref|Homo sapiens calcium/calmodulin-dependent protein kinase IG (CAMK1G), mRNA [NM_020439]	6,75
RAB27A	NM_004580	ref|Homo sapiens RAB27A, member RAS oncogene family (RAB27A), transcript variant 1, mRNA [NM_004580]	6,72
CHRDL2	NM_015424	ref|Homo sapiens chordin-like 2 (CHRDL2), mRNA [NM_015424]	6,71
FGFBP2	NM_031950	ref|Homo sapiens fibroblast growth factor binding protein 2 (FGFBP2), mRNA [NM_031950]	6,70
CEACAM1	NM_001024912	ref|Homo sapiens carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein) (CEACAM1), transcript variant 2, mRNA [NM_001024912]	6,69
CA14	NM_012113	ref|Homo sapiens carbonic anhydrase XIV (CA14), mRNA [NM_012113]	6,64
D4S234E	NM_014392	ref|Homo sapiens DNA segment on chromosome 4 (unique) 234 expressed sequence (D4S234E), transcript variant 1, mRNA [NM_014392]	6,60
PPY2	NR_002181	ref|Homo sapiens pancreatic polypeptide 2 (PPY2), non-coding RNA [NR_002181]	6,57
BFSP2	NM_003571	ref|Homo sapiens beaded filament structural protein 2, phakinin (BFSP2), mRNA [NM_003571]	6,57
CAPN3	NM_000070	ref|Homo sapiens calpain 3, (p94) (CAPN3), transcript variant 1, mRNA [NM_000070]	6,56
IGF1	NM_000618	ref|Homo sapiens insulin-like growth factor 1 (somatomedin C) (IGF1), transcript variant 4, mRNA [NM_000618]	6,55
PLB1	NM_153021	ref|Homo sapiens phospholipase B1 (PLB1), mRNA [NM_153021]	6,53
SPACA3	NM_173847	ref|Homo sapiens sperm acrosome associated 3 (SPACA3), mRNA [NM_173847]	6,49

C21orf129	AK057397	gb|Homo sapiens cDNA FLJ32835 fis, clone TESTI2003255. [AK057397]	6,47
RP13-401N8.2	AL096727	gb|Homo sapiens mRNA; cDNA DKFZp434B104 (from clone DKFZp434B104). [AL096727]	6,46
ST6GALNAC2	NM_006456	ref|Homo sapiens ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2 (ST6GALNAC2), mRNA [NM_(	6,42
PLP1	BC002665	gb|Homo sapiens proteolipid protein 1, mRNA (cDNA clone MGC:3940 IMAGE:3606269), complete cds. [BC002665]	6,36
FGFBP2	NM_031950	ref|Homo sapiens fibroblast growth factor binding protein 2 (FGFBP2), mRNA [NM_031950]	6,34
PRDM9	NM_020227	ref|Homo sapiens PR domain containing 9 (PRDM9), mRNA [NM_020227]	6,32
PDE4DIP	NM_014644	ref|Homo sapiens phosphodiesterase 4D interacting protein (PDE4DIP), transcript variant 1, mRNA [NM_014644]	6,29
BC014938	BC014938	gb|Homo sapiens, clone IMAGE:3922198, mRNA. [BC014938]	6,23
C1orf54	NM_024579	ref|Homo sapiens chromosome 1 open reading frame 54 (C1orf54), mRNA [NM_024579]	6,15
CARD14	NM_024110	ref|Homo sapiens caspase recruitment domain family, member 14 (CARD14), transcript variant 1, mRNA [NM_024110]	6,15
LUZP4	NM_016383	ref|Homo sapiens leucine zipper protein 4 (LUZP4), mRNA [NM_016383]	6,12
DEXI	AK055529	gb|Homo sapiens cDNA FLJ30967 fis, clone HEART2000309, weakly similar to PTB-ASSOCIATED SPLICING FACTOR. [AK055529]	6,02
DSP	NM_004415	ref|Homo sapiens desmoplakin (DSP), transcript variant 1, mRNA [NM_004415]	6,00
SCGB1D2	NM_006551	ref|Homo sapiens secretoglobin, family 1D, member 2 (SCGB1D2), mRNA [NM_006551]	5,92
ITGA7	NM_002206	ref|Homo sapiens integrin, alpha 7 (ITGA7), mRNA [NM_002206]	5,92
BIRC7	NM_022161	ref|Homo sapiens baculoviral IAP repeat-containing 7 (BIRC7), transcript variant 2, mRNA [NM_022161]	5,85
DYNC1I1	NM_004411	ref|Homo sapiens dynein, cytoplasmic 1, intermediate chain 1 (DYNC1I1), transcript variant 1, mRNA [NM_004411]	5,76
MGP	NM_000900	ref|Homo sapiens matrix Gla protein (MGP), mRNA [NM_000900]	5,67
MYLK	NM_053025	ref|Homo sapiens myosin light chain kinase (MYLK), transcript variant 1, mRNA [NM_053025]	5,67
MLLT10	BC032532	gb|Homo sapiens myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 10, mRNA (cDNA clone IMAGE:5526778), with a	5,66
THC2689164	THC2689164	Unknown	5,65
HBG1	NM_000559	ref|Homo sapiens hemoglobin, gamma A (HBG1), mRNA [NM_000559]	5,65
SEMA6A	NM_020796	ref|Homo sapiens sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A (SEMA6A), mRNA [NM_020796]	5,63
VGF	NM_003378	ref|Homo sapiens VGF nerve growth factor inducible (VGF), mRNA [NM_003378]	5,58
LOC645591	BC012751	gb|Homo sapiens cDNA clone IMAGE:3621376, partial cds. [BC012751]	5,56
HAS2	NM_005328	ref|Homo sapiens hyaluronan synthase 2 (HAS2), mRNA [NM_005328]	5,52
TRIM63	NM_032588	ref|Homo sapiens tripartite motif-containing 63 (TRIM63), mRNA [NM_032588]	5,52
SULT1C2	NM_176825	ref|Homo sapiens sulfotransferase family, cytosolic, 1C, member 2 (SULT1C2), transcript variant 2, mRNA [NM_176825]	5,46
LRAT	NM_004744	ref|Homo sapiens lecithin retinol acyltransferase (phosphatidylcholine--retinol O-acyltransferase) (LRAT), mRNA [NM_004744]	5,46
BHLHE41	NM_030762	ref|Homo sapiens basic helix-loop-helix domain containing, class B, 3 (BHLHB3), mRNA [NM_030762]	5,40
THC2733597	THC2733597	thc|Q40J89_EHRCH (Q40J89) Cation efflux protein, partial (6%) [THC2733597]	5,33
THC2701341	THC2701341	thc|DQ071571 spermatin {Rattus norvegicus} (exp=-1; wgp=0; cg=0), partial (19%) [THC2701341]	5,28
SEMA6A	NM_020796	ref|Homo sapiens sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A (SEMA6A), mRNA [NM_020796]	5,27
SGCD	NM_000337	ref|Homo sapiens sarcoglycan, delta (35kDa dystrophin-associated glycoprotein) (SGCD), transcript variant 1, mRNA [NM_000337]	5,25
hCG_2019139	AK056971	gb|Homo sapiens cDNA FLJ32409 fis, clone SKMUS2000468. [AK056971]	5,23
AF339787	AF339787	gb|Homo sapiens clone IMAGE:205688, mRNA sequence. [AF339787]	5,18
CBX5	NM_001127322	ref|Homo sapiens chromobox homolog 5 (HP1 alpha homolog, Drosophila) (CBX5), transcript variant 1, mRNA [NM_001127322]	5,18
ST3GAL4	NM_006278	ref|Homo sapiens ST3 beta-galactoside alpha-2,3-sialyltransferase 4 (ST3GAL4), mRNA [NM_006278]	5,17
BCAN	NM_198427	ref|Homo sapiens brevican (BCAN), transcript variant 2, mRNA [NM_198427]	5,13
CTSZ	NM_001336	ref|Homo sapiens cathepsin Z (CTSZ), mRNA [NM_001336]	5,12
MITF	NM_198159	ref|Homo sapiens microphthalmia-associated transcription factor (MITF), transcript variant 1, mRNA [NM_198159]	5,11
ATOH8	NM_032827	ref|Homo sapiens atonal homolog 8 (Drosophila) (ATOH8), mRNA [NM_032827]	5,08
MITF	NM_198159	ref|Homo sapiens microphthalmia-associated transcription factor (MITF), transcript variant 1, mRNA [NM_198159]	5,07
GALNT3	NM_004482	ref|Homo sapiens UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3) (GALNT3), mRNA [NM_004482]	5,01
CDK2	NM_001798	ref|Homo sapiens cyclin-dependent kinase 2 (CDK2), transcript variant 1, mRNA [NM_001798]	4,98
TYRP1	NM_000550	ref|Homo sapiens tyrosinase-related protein 1 (TYRP1), mRNA [NM_000550]	4,98
LOC286382	AK094356	gb|Homo sapiens cDNA FLJ37037 fis, clone BRACE2011611. [AK094356]	4,97
DYNC1I1	NM_004411	ref|Homo sapiens dynein, cytoplasmic 1, intermediate chain 1 (DYNC1I1), transcript variant 1, mRNA [NM_004411]	4,96
PMP2	NM_002677	ref|Homo sapiens peripheral myelin protein 2 (PMP2), mRNA [NM_002677]	4,89
BACE2	NM_012105	ref|Homo sapiens beta-site APP-cleaving enzyme 2 (BACE2), transcript variant a, mRNA [NM_012105]	4,87
SOX10	NM_006941	ref|Homo sapiens SRY (sex determining region Y)-box 10 (SOX10), mRNA [NM_006941]	4,85
SOX10	NM_006941	ref|Homo sapiens SRY (sex determining region Y)-box 10 (SOX10), mRNA [NM_006941]	4,85
CDK2	NM_001798	ref|Homo sapiens cyclin-dependent kinase 2 (CDK2), transcript variant 1, mRNA [NM_001798]	4,83
CDK2	NM_001798	ref|Homo sapiens cyclin-dependent kinase 2 (CDK2), transcript variant 1, mRNA [NM_001798]	4,82
MYLK	NM_053025	ref|Homo sapiens myosin light chain kinase (MYLK), transcript variant 1, mRNA [NM_053025]	4,82
PHF15	NM_015288	ref|Homo sapiens PHD finger protein 15 (PHF15), mRNA [NM_015288]	4,81
GPR161	NM_153832	ref|Homo sapiens G protein-coupled receptor 161 (GPR161), transcript variant 2, mRNA [NM_153832]	4,81
PHF15	NM_015288	ref|Homo sapiens PHD finger protein 15 (PHF15), mRNA [NM_015288]	4,80
C16orf89	NM_152459	ref|Homo sapiens chromosome 16 open reading frame 89 (C16orf89), transcript variant 1, mRNA [NM_152459]	4,80
SIRPB1	NM_006065	ref|Homo sapiens signal-regulatory protein beta 1 (SIRPB1), transcript variant 1, mRNA [NM_006065]	4,79
BACE2	NM_012105	ref|Homo sapiens beta-site APP-cleaving enzyme 2 (BACE2), transcript variant a, mRNA [NM_012105]	4,78
SIRPA	NM_001040022	ref|Homo sapiens signal-regulatory protein alpha (SIRPA), transcript variant 1, mRNA [NM_001040022]	4,78
KCNAB1	NM_003471	ref|Homo sapiens potassium voltage-gated channel, shaker-related subfamily, beta member 1 (KCNAB1), transcript variant 2, mRNA [NM_003471]	4,78
CDK2	NM_001798	ref|Homo sapiens cyclin-dependent kinase 2 (CDK2), transcript variant 1, mRNA [NM_001798]	4,76
PKD1L2	NM_052892	ref|Homo sapiens polycystic kidney disease 1-like 2 (PKD1L2), transcript variant 1, mRNA [NM_052892]	4,75
HBG1	NM_000559	ref|Homo sapiens hemoglobin, gamma A (HBG1), mRNA [NM_000559]	4,75
CDK2	NM_001798	ref|Homo sapiens cyclin-dependent kinase 2 (CDK2), transcript variant 1, mRNA [NM_001798]	4,75
KCNAB2	NM_003636	ref|Homo sapiens potassium voltage-gated channel, shaker-related subfamily, beta member 2 (KCNAB2), transcript variant 1, mRNA [NM_003636]	4,72
PPP1CB	NM_002709	ref|Homo sapiens protein phosphatase 1, catalytic subunit, beta isoform (PPP1CB), transcript variant 1, mRNA [NM_002709]	4,72
C6orf218	NM_152738	ref|Homo sapiens chromosome 6 open reading frame 218 (C6orf218), mRNA [NM_152738]	4,70
ENST00000327299	ENST00000327299	ens|Adenylate kinase isoenzyme 4, mitochondrial (EC 2.7.4.3)(Adenylate kinase 3-like)(ATP-AMP transphosphorylase) [Source:UniProtKB/Swiss-Prot;Acc:P271	4,70
GHR	NM_000163	ref|Homo sapiens growth hormone receptor (GHR), mRNA [NM_000163]	4,68
PPARGC1A	NM_013261	ref|Homo sapiens peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC1A), mRNA [NM_013261]	4,67
HBG1	NM_000559	ref|Homo sapiens hemoglobin, gamma A (HBG1), mRNA [NM_000559]	4,67
SLIT1	NM_003061	ref|Homo sapiens slit homolog 1 (Drosophila) (SLIT1), mRNA [NM_003061]	4,66
PTCRA	NM_138296	ref|Homo sapiens pre T-cell antigen receptor alpha (PTCRA), mRNA [NM_138296]	4,64
MOBKL2B	NM_024761	ref|Homo sapiens MOB1, Mps One Binder kinase activator-like 2B (yeast) (MOBKL2B), mRNA [NM_024761]	4,62
CDK2	NM_001798	ref|Homo sapiens cyclin-dependent kinase 2 (CDK2), transcript variant 1, mRNA [NM_001798]	4,61
MLPH	NM_024101	ref|Homo sapiens melanophilin (MLPH), transcript variant 1, mRNA [NM_024101]	4,61
GJB1	NM_000166	ref|Homo sapiens gap junction protein, beta 1, 32kDa (GJB1), transcript variant 2, mRNA [NM_000166]	4,60
MCAM	NM_006500	ref|Homo sapiens melanoma cell adhesion molecule (MCAM), mRNA [NM_006500]	4,59
CDK2	NM_001798	ref|Homo sapiens cyclin-dependent kinase 2 (CDK2), transcript variant 1, mRNA [NM_001798]	4,59
LEPREL1	NM_018192	ref|Homo sapiens leprecan-like 1 (LEPREL1), transcript variant 1, mRNA [NM_018192]	4,59
MITF	NM_198159	ref|Homo sapiens microphthalmia-associated transcription factor (MITF), transcript variant 1, mRNA [NM_198159]	4,58
ENO2	NM_001975	ref|Homo sapiens enolase 2 (gamma, neuronal) (ENO2), mRNA [NM_001975]	4,58
DHRS2	NM_182908	ref|Homo sapiens dehydrogenase/reductase (SDR family) member 2 (DHRS2), transcript variant 1, mRNA [NM_182908]	4,58
HBG1	NM_000559	ref|Homo sapiens hemoglobin, gamma A (HBG1), mRNA [NM_000559]	4,57
CDK2	NM_001798	ref|Homo sapiens cyclin-dependent kinase 2 (CDK2), transcript variant 1, mRNA [NM_001798]	4,55
CV800149	CV800149	gb|AGENCOURT_37001201 NIH_MGC_280 Homo sapiens cDNA clone IMAGE:7505458 5', mRNA sequence [CV800149]	4,49
SPRR3	NM_005416	ref|Homo sapiens small proline-rich protein 3 (SPRR3), transcript variant 1, mRNA [NM_005416]	4,48
ITK	NM_005546	ref|Homo sapiens IL2-inducible T-cell kinase (ITK), mRNA [NM_005546]	4,48
KIAA0746	NM_015187	ref|Homo sapiens KIAA0746 protein (KIAA0746), mRNA [NM_015187]	4,48
THC2520542	THC2520542	thc|Q9XDH2_MYCTU (Q9XDH2) Proline-rich mucin homolog, partial (3%) [THC2520542]	4,48
ATP10A	NM_024490	ref|Homo sapiens ATPase, class V, type 10A (ATP10A), mRNA [NM_024490]	4,46
ST3GAL4	AK021929	gb|Homo sapiens cDNA FLJ11867 fis, clone HEMBA1006976, weakly similar to H.sapiens mRNA for Gal-beta(1-3/1-4)GlcNAc alpha-2.3-sialyltransferase. [AK02	4,45
HBG1	NM_000559	ref|Homo sapiens hemoglobin, gamma A (HBG1), mRNA [NM_000559]	4,44
GJB1	NM_000166	ref|Homo sapiens gap junction protein, beta 1, 32kDa (GJB1), transcript variant 2, mRNA [NM_000166]	4,44
MYH10	NM_005964	ref|Homo sapiens myosin, heavy chain 10, non-muscle (MYH10), mRNA [NM_005964]	4,43
DUSP23	NM_017823	ref|Homo sapiens dual specificity phosphatase 23 (DUSP23), mRNA [NM_017823]	4,43
CDK2	NM_001798	ref|Homo sapiens cyclin-dependent kinase 2 (CDK2), transcript variant 1, mRNA [NM_001798]	4,43
PKIA	NM_006823	ref|Homo sapiens protein kinase (cAMP-dependent, catalytic) inhibitor alpha (PKIA), transcript variant 6, mRNA [NM_006823]	4,40
RGN	NM_152869	ref|Homo sapiens regucalcin (senescence marker protein-30) (RGN), transcript variant 2, mRNA [NM_152869]	4,40
CCL17	NM_002987	ref|Homo sapiens chemokine (C-C motif) ligand 17 (CCL17), mRNA [NM_002987]	4,38
OPHN1	NM_002547	ref|Homo sapiens oligophrenin 1 (OPHN1), mRNA [NM_002547]	4,38
FAM174B	NM_207446	ref|Homo sapiens family with sequence similarity 174, member B (FAM174B), mRNA [NM_207446]	4,37
FSTL5	NM_020116	ref|Homo sapiens follistatin-like 5 (FSTL5), transcript variant 1, mRNA [NM_020116]	4,34
MCAM	NM_006500	ref|Homo sapiens melanoma cell adhesion molecule (MCAM), mRNA [NM_006500]	4,34
HBG1	NM_000559	ref|Homo sapiens hemoglobin, gamma A (HBG1), mRNA [NM_000559]	4,32
DHRS2	NM_182908	ref|Homo sapiens dehydrogenase/reductase (SDR family) member 2 (DHRS2), transcript variant 1, mRNA [NM_182908]	4,32
ST6GALNAC1	NM_018414	ref|Homo sapiens ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1 (ST6GALNAC1), mRNA [NM_(	4,29
NAT8B	NM_016347	ref|Homo sapiens N-acetyltransferase 8B (GCN5-related, putative, gene/pseudogene) (NAT8B), mRNA [NM_016347]	4,26

PMP2	NM_002677	ref|Homo sapiens peripheral myelin protein 2 (PMP2), mRNA [NM_002677]	4,26
HBG1	NM_000559	ref|Homo sapiens hemoglobin, gamma A (HBG1), mRNA [NM_000559]	4,25
GAGE3	U19144	gb|Human GAGE-3 protein mRNA, complete cds. [U19144]	4,25
LDB3	NM_007078	ref|Homo sapiens LIM domain binding 3 (LDB3), transcript variant 1, mRNA [NM_007078]	4,24
CDK2	NM_001798	ref|Homo sapiens cyclin-dependent kinase 2 (CDK2), transcript variant 1, mRNA [NM_001798]	4,24
FCRLA	NM_032738	ref|Homo sapiens Fc receptor-like A (FCRLA), mRNA [NM_032738]	4,24
BST2	NM_004335	ref|Homo sapiens bone marrow stromal cell antigen 2 (BST2), mRNA [NM_004335]	4,22
AP1S2	NM_003916	ref|Homo sapiens adaptor-related protein complex 1, sigma 2 subunit (AP1S2), mRNA [NM_003916]	4,22
SILV	NM_006928	ref|Homo sapiens silver homolog (mouse) (SILV), mRNA [NM_006928]	4,21
SIRPA	NM_001040022	ref|Homo sapiens signal-regulatory protein alpha (SIRPA), transcript variant 1, mRNA [NM_001040022]	4,19
MLPH	NM_001042467	ref|Homo sapiens melanophilin (MLPH), transcript variant 2, mRNA [NM_001042467]	4,19
CR749704	CR749704	gb|Homo sapiens mRNA; cDNA DKFZp434D1072 (from clone DKFZp434D1072). [CR749704]	4,17
BC063881	BC063881	gb|Homo sapiens cDNA clone IMAGE:30390303, with apparent retained intron. [BC063881]	4,14
SLC45A2	NM_016180	ref|Homo sapiens solute carrier family 45, member 2 (SLC45A2), transcript variant 1, mRNA [NM_016180]	4,12
HBG1	NM_000559	ref|Homo sapiens hemoglobin, gamma A (HBG1), mRNA [NM_000559]	4,11
DMKN	NM_001035516	ref|Homo sapiens dermokine (DMKN), transcript variant 1, mRNA [NM_001035516]	4,10
STK32A	NM_145001	ref|Homo sapiens serine/threonine kinase 32A (STK32A), transcript variant 2, mRNA [NM_145001]	4,09
FAM167B	NM_032648	ref|Homo sapiens family with sequence similarity 167, member B (FAM167B), mRNA [NM_032648]	4,08
AK024680	AK024680	gb|Homo sapiens cDNA: FLJ21027 fis, clone CAE07110. [AK024680]	4,05
MOBKL2B	NM_024761	ref|Homo sapiens MOB1, Mps One Binder kinase activator-like 2B (yeast) (MOBKL2B), mRNA [NM_024761]	4,04
UROS	CR601670	gb|full-length cDNA clone CS0DL001YD09 of B cells (Ramos cell line) Cot 25-normalized of Homo sapiens (human). [CR601670]	4,04
VEGFB	NM_003377	ref|Homo sapiens vascular endothelial growth factor B (VEGFB), mRNA [NM_003377]	4,04
TTC39A	NM_001080494	ref|Homo sapiens tetratricopeptide repeat domain 39A (TTC39A), mRNA [NM_001080494]	4,03
HBG1	NM_000559	ref|Homo sapiens hemoglobin, gamma A (HBG1), mRNA [NM_000559]	4,02
CEACAM3	NM_001815	ref|Homo sapiens carcinoembryonic antigen-related cell adhesion molecule 3 (CEACAM3), mRNA [NM_001815]	4,01
HBG1	NM_000559	ref|Homo sapiens hemoglobin, gamma A (HBG1), mRNA [NM_000559]	4,00
GPR161	NM_153832	ref|Homo sapiens G protein-coupled receptor 161 (GPR161), transcript variant 2, mRNA [NM_153832]	4,00
ITM2A	NM_004867	ref|Homo sapiens integral membrane protein 2A (ITM2A), mRNA [NM_004867]	4,00
LRAT	NM_004744	ref|Homo sapiens lecithin retinol acyltransferase (phosphatidylcholine--retinol O-acyltransferase) (LRAT), mRNA [NM_004744]	3,99
MLPH	NM_024101	ref|Homo sapiens melanophilin (MLPH), transcript variant 1, mRNA [NM_024101]	3,98
FUT5	NM_002034	ref|Homo sapiens fucosyltransferase 5 (alpha (1,3) fucosyltransferase) (FUT5), mRNA [NM_002034]	3,97
GPR56	NM_201525	ref|Homo sapiens G protein-coupled receptor 56 (GPR56), transcript variant 3, mRNA [NM_201525]	3,96
PPM1H	NM_020700	ref|Homo sapiens protein phosphatase 1H (PP2C domain containing) (PPM1H), mRNA [NM_020700]	3,95
HBG1	NM_000559	ref|Homo sapiens hemoglobin, gamma A (HBG1), mRNA [NM_000559]	3,94
ME3	NM_001014811	ref|Homo sapiens malic enzyme 3, NADP(+)-dependent, mitochondrial (ME3), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA [NM_001	3,93
GPR161	NM_153832	ref|Homo sapiens G protein-coupled receptor 161 (GPR161), transcript variant 2, mRNA [NM_153832]	3,92
E2F1	NM_005225	ref|Homo sapiens E2F transcription factor 1 (E2F1), mRNA [NM_005225]	3,92
ENST00000327299	ENST00000327299	ens|Adenylate kinase isoenzyme 4, mitochondrial (EC 2.7.4.3)(Adenylate kinase 3-like)(ATP-AMP transphosphorylase) [Source:UniProtKB/Swiss-Prot;Acc:P271	3,91
AX721193	AX721193	gb|Sequence 153 from Patent WO0220754. [AX721193]	3,91
MDGA1	NM_153487	ref|Homo sapiens MAM domain containing glycosylphosphatidylinositol anchor 1 (MDGA1), mRNA [NM_153487]	3,91
SMPD1	NM_000543	ref|Homo sapiens sphingomyelin phosphodiesterase 1, acid lysosomal (SMPD1), transcript variant 1, mRNA [NM_000543]	3,89
E2F1	NM_005225	ref|Homo sapiens E2F transcription factor 1 (E2F1), mRNA [NM_005225]	3,89
ANXA13	NM_001003954	ref|Homo sapiens annexin A13 (ANXA13), transcript variant 2, mRNA [NM_001003954]	3,88
BI013473	BI013473	gb|RC0-ET0185-220101-021-h02 ET0185 Homo sapiens cDNA, mRNA sequence [BI013473]	3,88
E2F1	NM_005225	ref|Homo sapiens E2F transcription factor 1 (E2F1), mRNA [NM_005225]	3,87
BC061638	BC061638	gb|Homo sapiens cDNA clone IMAGE:5547707, partial cds. [BC061638]	3,86
E2F1	NM_005225	ref|Homo sapiens E2F transcription factor 1 (E2F1), mRNA [NM_005225]	3,86
STXBP6	NM_014178	ref|Homo sapiens syntaxin binding protein 6 (amisyn) (STXBP6), mRNA [NM_014178]	3,85
LRGUK	NM_144648	ref|Homo sapiens leucine-rich repeats and guanylate kinase domain containing (LRGUK), mRNA [NM_144648]	3,85
GAGE7	NM_021123	ref|Homo sapiens G antigen 7 (GAGE7), mRNA [NM_021123]	3,84
MORC1	NM_014429	ref|Homo sapiens MORC family CW-type zinc finger 1 (MORC1), mRNA [NM_014429]	3,84
PTPRJ	NM_002843	ref|Homo sapiens protein tyrosine phosphatase, receptor type, J (PTPRJ), transcript variant 1, mRNA [NM_002843]	3,84
E2F1	NM_005225	ref|Homo sapiens E2F transcription factor 1 (E2F1), mRNA [NM_005225]	3,83
FYB	NM_001465	ref|Homo sapiens FYN binding protein (FYB-120/130) (FYB), transcript variant 1, mRNA [NM_001465]	3,83
AK090694	AK090694	gb|Homo sapiens cDNA FLJ33375 fis, clone BRACE2006137. [AK090694]	3,83
E2F1	NM_005225	ref|Homo sapiens E2F transcription factor 1 (E2F1), mRNA [NM_005225]	3,83
CBLN1	NM_004352	ref|Homo sapiens cerebellin 1 precursor (CBLN1), mRNA [NM_004352]	3,82
GHR	NM_000163	ref|Homo sapiens growth hormone receptor (GHR), mRNA [NM_000163]	3,82
SCARB1	NM_005505	ref|Homo sapiens scavenger receptor class B, member 1 (SCARB1), transcript variant 1, mRNA [NM_005505]	3,81
PRDM9	NM_020227	ref|Homo sapiens PR domain containing 9 (PRDM9), mRNA [NM_020227]	3,79
HLA-DMB	NM_002118	ref|Homo sapiens major histocompatibility complex, class II, DM beta (HLA-DMB), mRNA [NM_002118]	3,79
DHRS3	NM_004753	ref|Homo sapiens dehydrogenase/reductase (SDR family) member 3 (DHRS3), mRNA [NM_004753]	3,77
PKD1L2	NM_052892	ref|Homo sapiens polycystic kidney disease 1-like 2 (PKD1L2), transcript variant 1, mRNA [NM_052892]	3,77
SNAI2	NM_003068	ref|Homo sapiens snail homolog 2 (Drosophila) (SNAI2), mRNA [NM_003068]	3,76
E2F1	NM_005225	ref|Homo sapiens E2F transcription factor 1 (E2F1), mRNA [NM_005225]	3,76
E2F1	NM_005225	ref|Homo sapiens E2F transcription factor 1 (E2F1), mRNA [NM_005225]	3,76
ITGB8	NM_002214	ref|Homo sapiens integrin, beta 8 (ITGB8), mRNA [NM_002214]	3,75
EDEM1	NM_014674	ref|Homo sapiens ER degradation enhancer, mannosidase alpha-like 1 (EDEM1), mRNA [NM_014674]	3,75
RGS20	NM_170587	ref|Homo sapiens regulator of G-protein signaling 20 (RGS20), transcript variant 1, mRNA [NM_170587]	3,73
CABLES1	NM_138375	ref|Homo sapiens Cdk5 and Abl enzyme substrate 1 (CABLES1), transcript variant 1, mRNA [NM_138375]	3,73
IRF4	NM_002460	ref|Homo sapiens interferon regulatory factor 4 (IRF4), mRNA [NM_002460]	3,72
E2F1	NM_005225	ref|Homo sapiens E2F transcription factor 1 (E2F1), mRNA [NM_005225]	3,71
E2F1	NM_005225	ref|Homo sapiens E2F transcription factor 1 (E2F1), mRNA [NM_005225]	3,71
EPHX1	NM_000120	ref|Homo sapiens epoxide hydrolase 1, microsomal (xenobiotic) (EPHX1), transcript variant 1, mRNA [NM_000120]	3,71
GPR56	NM_201525	ref|Homo sapiens G protein-coupled receptor 56 (GPR56), transcript variant 3, mRNA [NM_201525]	3,69
LYPD1	NM_144586	ref|Homo sapiens LY6/PLAUR domain containing 1 (LYPD1), transcript variant 1, mRNA [NM_144586]	3,69
FAM152A	BC020640	gb|Homo sapiens family with sequence similarity 152, member A, mRNA (cDNA clone IMAGE:4718788), partial cds. [BC020640]	3,69
KRT23	NM_015515	ref|Homo sapiens keratin 23 (histone deacetylase inducible) (KRT23), mRNA [NM_015515]	3,68
PTTG1IP	NM_004339	ref|Homo sapiens pituitary tumor-transforming 1 interacting protein (PTTG1IP), mRNA [NM_004339]	3,68
X83127	X83127	gb|H.sapiens mRNA for voltage gated potassium channels, beta subunit. [X83127]	3,68
PLXNC1	AB208934	gb|Homo sapiens mRNA for plexin C1 variant protein. [AB208934]	3,68
TMPRSS5	NM_030770	ref|Homo sapiens transmembrane protease, serine 5 (TMPRSS5), mRNA [NM_030770]	3,68
SMOC2	NM_022138	ref|Homo sapiens SPARC related modular calcium binding 2 (SMOC2), mRNA [NM_022138]	3,67
NRP2	NM_201266	ref|Homo sapiens neuropilin 2 (NRP2), transcript variant 1, mRNA [NM_201266]	3,67
BG682198	BG682198	gb|602629505F1 NCI_CGAP_Skn4 Homo sapiens cDNA clone IMAGE:4754377 5', mRNA sequence [BG682198]	3,67
SFTPC	NM_003018	ref|Homo sapiens surfactant protein C (SFTPC), mRNA [NM_003018]	3,67
C15orf39	NM_015492	ref|Homo sapiens chromosome 15 open reading frame 39 (C15orf39), mRNA [NM_015492]	3,66
AFAP1L1	NM_152406	ref|Homo sapiens actin filament associated protein 1-like 1 (AFAP1L1), mRNA [NM_152406]	3,66
CDH7	NM_004361	ref|Homo sapiens cadherin 7, type 2 (CDH7), transcript variant b, mRNA [NM_004361]	3,65
DENND2A	NM_015689	ref|Homo sapiens DENN/MADD domain containing 2A (DENND2A), mRNA [NM_015689]	3,65
S1PR4	NM_003775	ref|Homo sapiens sphingosine-1-phosphate receptor 4 (S1PR4), mRNA [NM_003775]	3,65
OR7A5	NM_017506	ref|Homo sapiens olfactory receptor, family 7, subfamily A, member 5 (OR7A5), mRNA [NM_017506]	3,64
KEL	NM_000420	ref|Homo sapiens Kell blood group, metallo-endopeptidase (KEL), mRNA [NM_000420]	3,64
C9orf93	NM_173550	ref|Homo sapiens chromosome 9 open reading frame 93 (C9orf93), mRNA [NM_173550]	3,63
LOC390595	AK023802	gb|Homo sapiens cDNA FLJ13740 fis, clone PLACE3000199. [AK023802]	3,61
THC2689504	THC2689504	thc|Q5E4L1_VIBF1 (Q5E4L1) Suppressor/enhancer of lin-12, partial (6%) [THC2689504]	3,61
AA778291	AA778291	gb|AA778291 zl56d07.s1 Soares_pregnant_uterus_NbHPU Homo sapiens cDNA clone IMAGE:505933 3', mRNA sequence [AA778291]	3,60
WHSC1	NM_007331	ref|Homo sapiens Wolf-Hirschhorn syndrome candidate 1 (WHSC1), transcript variant 8, mRNA [NM_007331]	3,60
OLFM1	NM_014279	ref|Homo sapiens olfactomedin 1 (OLFM1), transcript variant 1, mRNA [NM_014279]	3,59
LGI3	NM_139278	ref|Homo sapiens leucine-rich repeat LGI family, member 3 (LGI3), mRNA [NM_139278]	3,58
CARD14	NM_052819	ref|Homo sapiens caspase recruitment domain family, member 14 (CARD14), transcript variant 2, mRNA [NM_052819]	3,57
AP1S2	NM_003916	ref|Homo sapiens adaptor-related protein complex 1, sigma 2 subunit (AP1S2), mRNA [NM_003916]	3,56
SSU72	AK023110	gb|Homo sapiens cDNA FLJ13048 fis, clone NT2RP3001399, weakly similar to SSU72 PROTEIN. [AK023110]	3,54
SCARB1	NM_005505	ref|Homo sapiens scavenger receptor class B, member 1 (SCARB1), transcript variant 1, mRNA [NM_005505]	3,53
NAV2	NM_182964	ref|Homo sapiens neuron navigator 2 (NAV2), transcript variant 1, mRNA [NM_182964]	3,51
PPARGC1A	NM_013261	ref|Homo sapiens peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC1A), mRNA [NM_013261]	3,51
CNIH3	NM_152495	ref|Homo sapiens cornichon homolog 3 (Drosophila) (CNIH3), mRNA [NM_152495]	3,50
TYR	NM_000372	ref|Homo sapiens tyrosinase (oculocutaneous albinism IA) (TYR), mRNA [NM_000372]	3,49
FSTL4	NM_015082	ref|Homo sapiens follistatin-like 4 (FSTL4), mRNA [NM_015082]	3,48
NCALD	NM_001040630	ref|Homo sapiens neurocalcin delta (NCALD), transcript variant 7, mRNA [NM_001040630]	3,47
VEGFB	NM_003377	ref|Homo sapiens vascular endothelial growth factor B (VEGFB), mRNA [NM_003377]	3,44

AK021785	AK021785	gb|Homo sapiens cDNA FLJ11723 fis, clone HEMBA1005314. [AK021785]	3,44
CPB1	NM_001871	ref|Homo sapiens carboxypeptidase B1 (tissue) (CPB1), mRNA [NM_001871]	3,43
HSPB2	NM_001541	ref|Homo sapiens heat shock 27kDa protein 2 (HSPB2), mRNA [NM_001541]	3,42
NR4A3	NM_173198	ref|Homo sapiens nuclear receptor subfamily 4, group A, member 3 (NR4A3), transcript variant 2, mRNA [NM_173198]	3,41
GAS7	NM_201433	ref|Homo sapiens growth arrest-specific 7 (GAS7), transcript variant c, mRNA [NM_201433]	3,41
AF086187	AF086187	gb|Homo sapiens full length insert cDNA clone ZC30H06. [AF086187]	3,41
IGSF8	NM_052868	ref|Homo sapiens immunoglobulin superfamily, member 8 (IGSF8), mRNA [NM_052868]	3,41
DHRS13	NM_144683	ref|Homo sapiens dehydrogenase/reductase (SDR family) member 13 (DHRS13), mRNA [NM_144683]	3,39
SEMA7A	NM_003612	ref|Homo sapiens semaphorin 7A, GPI membrane anchor (John Milton Hagen blood group) (SEMA7A), mRNA [NM_003612]	3,38
MBOAT1	NM_001080480	ref|Homo sapiens membrane bound O-acyltransferase domain containing 1 (MBOAT1), mRNA [NM_001080480]	3,38
SILV	NM_006928	ref|Homo sapiens silver homolog (mouse) (SILV), mRNA [NM_006928]	3,37
ATP8A2	NM_016529	ref|Homo sapiens ATPase, aminophospholipid transporter-like, class I, type 8A, member 2 (ATP8A2), mRNA [NM_016529]	3,37
KIAA1598	NM_018330	ref|Homo sapiens KIAA1598 (KIAA1598), transcript variant 2, mRNA [NM_018330]	3,37
OSBPL10	NM_017784	ref|Homo sapiens oxysterol binding protein-like 10 (OSBPL10), mRNA [NM_017784]	3,37
SLAMF8	NM_020125	ref|Homo sapiens SLAM family member 8 (SLAMF8), mRNA [NM_020125]	3,36
ECM1	NM_004425	ref|Homo sapiens extracellular matrix protein 1 (ECM1), transcript variant 1, mRNA [NM_004425]	3,35
CFH	NM_001014975	ref|Homo sapiens complement factor H (CFH), transcript variant 2, mRNA [NM_001014975]	3,35
NAP5	NM_207363	ref|Homo sapiens Nck-associated protein 5 (NAP5), transcript variant 1, mRNA [NM_207363]	3,35
CA12	NM_001218	ref|Homo sapiens carbonic anhydrase XII (CA12), transcript variant 1, mRNA [NM_001218]	3,34
FLJ21075	NM_025031	ref|Homo sapiens hypothetical protein FLJ21075 (FLJ21075), mRNA [NM_025031]	3,33
C21orf90	NR_026547	ref|Homo sapiens chromosome 21 open reading frame 90 (C21orf90), transcript variant 1, non-coding RNA [NR_026547]	3,33
TYR	NM_000372	ref|Homo sapiens tyrosinase (oculocutaneous albinism IA) (TYR), mRNA [NM_000372]	3,33
RASSF4	NM_032023	ref|Homo sapiens Ras association (RalGDS/AF-6) domain family member 4 (RASSF4), mRNA [NM_032023]	3,32
FBXO2	NM_012168	ref|Homo sapiens F-box protein 2 (FBXO2), mRNA [NM_012168]	3,31
GDPD5	NM_030792	ref|Homo sapiens glycerophosphodiester phosphodiesterase domain containing 5 (GDPD5), mRNA [NM_030792]	3,31
JRK	NM_003724	ref|Homo sapiens jerky homolog (mouse) (JRK), transcript variant 1, mRNA [NM_003724]	3,31
ENST00000327299	ENST00000327299	ens|Adenylate kinase isoenzyme 4, mitochondrial (EC 2.7.4.3)(Adenylate kinase 3-like)(ATP-AMP transphosphorylase) [Source:UniProtKB/Swiss-Prot;Acc:P271	3,29
CITED1	NM_004143	ref|Homo sapiens Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 1 (CITED1), mRNA [NM_004143]	3,29
GDPD5	NM_030792	ref|Homo sapiens glycerophosphodiester phosphodiesterase domain containing 5 (GDPD5), mRNA [NM_030792]	3,28
FIGNL1	NM_001042762	ref|Homo sapiens fidgetin-like 1 (FIGNL1), transcript variant 1, mRNA [NM_001042762]	3,28
DYNLT3	NM_006520	ref|Homo sapiens dynein, light chain, Tctex-type 3 (DYNLT3), mRNA [NM_006520]	3,28
CKB	NM_001823	ref|Homo sapiens creatine kinase, brain (CKB), mRNA [NM_001823]	3,28
AMDHD2	NM_015944	ref|Homo sapiens amidohydrolase domain containing 2 (AMDHD2), mRNA [NM_015944]	3,27
TRPM3	NM_001007471	ref|Homo sapiens transient receptor potential cation channel, subfamily M, member 3 (TRPM3), transcript variant 9, mRNA [NM_001007471]	3,27
GPR143	NM_000273	ref|Homo sapiens G protein-coupled receptor 143 (GPR143), mRNA [NM_000273]	3,27
THC2539492	THC2539492	thc|CT091_HUMAN (Q5T1J6) Protein C20orf91, partial (66%) [THC2539492]	3,26
MAGEB6	NM_173523	ref|Homo sapiens melanoma antigen family B, 6 (MAGEB6), mRNA [NM_173523]	3,25
ENPP1	NM_006208	ref|Homo sapiens ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), mRNA [NM_006208]	3,25
PMP2	NM_002677	ref|Homo sapiens peripheral myelin protein 2 (PMP2), mRNA [NM_002677]	3,25
ATP6V0D2	NM_152565	ref|Homo sapiens ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2 (ATP6V0D2), mRNA [NM_152565]	3,24
CFH	NM_000186	ref|Homo sapiens complement factor H (CFH), transcript variant 1, mRNA [NM_000186]	3,24
C1orf133	NR_024337	ref|Homo sapiens chromosome 1 open reading frame 133 (C1orf133), non-coding RNA [NR_024337]	3,24
DENND2A	NM_015689	ref|Homo sapiens DENN/MADD domain containing 2A (DENND2A), mRNA [NM_015689]	3,23
MLLT10	NM_001009569	ref|Homo sapiens myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 10 (MLLT10), transcript variant 2, mRNA [NM_0(	3,23
ENST00000327506	ENST00000327506	ens|Protein LOC339766 [Source:UniProtKB/Swiss-Prot;Acc:A6NES4] [ENST00000327506]	3,22
BC047326	BC047326	gb|Homo sapiens, clone IMAGE:5528960, mRNA. [BC047326]	3,21
MAP7D2	NM_152780	ref|Homo sapiens MAP7 domain containing 2 (MAP7D2), mRNA [NM_152780]	3,20
NCRNA00084	U60873	gb|Human clone 137308 mRNA, partial cds. [U60873]	3,20
GYPC	NM_002101	ref|Homo sapiens glycophorin C (Gerbich blood group) (GYPC), transcript variant 1, mRNA [NM_002101]	3,19
FAM75A2	NM_001040065	ref|Homo sapiens family with sequence similarity 75, member A2 (FAM75A2), mRNA [NM_001040065]	3,19
STAC	NM_003149	ref|Homo sapiens SH3 and cysteine rich domain (STAC), mRNA [NM_003149]	3,18
CR620043	CR620043	gb|full-length cDNA clone CS0DC001YP21 of Neuroblastoma Cot 25-normalized of Homo sapiens (human). [CR620043]	3,18
SCML4	NM_198081	ref|Homo sapiens sex comb on midleg-like 4 (Drosophila) (SCML4), mRNA [NM_198081]	3,18
TNS3	NM_022748	ref|Homo sapiens tensin 3 (TNS3), mRNA [NM_022748]	3,18
ANKRD18A	BC131497	gb|Homo sapiens cDNA clone IMAGE:40081082. [BC131497]	3,17
PSMB10	NM_002801	ref|Homo sapiens proteasome (prosome, macropain) subunit, beta type, 10 (PSMB10), mRNA [NM_002801]	3,17
NBL1	NM_182744	ref|Homo sapiens neuroblastoma, suppression of tumorigenicity 1 (NBL1), transcript variant 1, mRNA [NM_182744]	3,17
THC2696414	THC2696414	Unknown	3,16
GINS2	NM_016095	ref|Homo sapiens GINS complex subunit 2 (Psf2 homolog) (GINS2), mRNA [NM_016095]	3,16
CTSA	NM_000308	ref|Homo sapiens cathepsin A (CTSA), transcript variant 1, mRNA [NM_000308]	3,16
GM2A	NM_000405	ref|Homo sapiens GM2 ganglioside activator (GM2A), mRNA [NM_000405]	3,16
CHAF1B	NM_005441	ref|Homo sapiens chromatin assembly factor 1, subunit B (p60) (CHAF1B), mRNA [NM_005441]	3,15
AT_ssH_GD_3	AT_ssH_GD_3		3,15
A2M	NM_000014	ref|Homo sapiens alpha-2-macroglobulin (A2M), mRNA [NM_000014]	3,14
TPCN2	NM_139075	ref|Homo sapiens two pore segment channel 2 (TPCN2), mRNA [NM_139075]	3,13
QDPR	M16447	gb|Human dihydropteridine reductase (hDHPR) mRNA, complete cds. [M16447]	3,13
CR618615	CR618615	gb|full-length cDNA clone CL0BB018ZH05 of Neuroblastoma of Homo sapiens (human). [CR618615]	3,13
THC2707492	THC2707492	Unknown	3,12
hCG_1774568	BC004179	gb|Homo sapiens similar to hCG1774568, mRNA (cDNA clone IMAGE:2959703), **** WARNING: chimeric clone ****. [BC004179]	3,11
P2RY2	NM_176072	ref|Homo sapiens purinergic receptor P2Y, G-protein coupled, 2 (P2RY2), transcript variant 1, mRNA [NM_176072]	3,10
DTNA	NM_001392	ref|Homo sapiens dystrobrevin, alpha (DTNA), transcript variant 7, mRNA [NM_001392]	3,09
BDH2	NM_020139	ref|Homo sapiens 3-hydroxybutyrate dehydrogenase, type 2 (BDH2), mRNA [NM_020139]	3,07
CHAF1B	NM_005441	ref|Homo sapiens chromatin assembly factor 1, subunit B (p60) (CHAF1B), mRNA [NM_005441]	3,07
CHAF1B	NM_005441	ref|Homo sapiens chromatin assembly factor 1, subunit B (p60) (CHAF1B), mRNA [NM_005441]	3,07
NAP5	NM_207481	ref|Homo sapiens Nck-associated protein 5 (NAP5), transcript variant 2, mRNA [NM_207481]	3,07
ACP5	NM_001611	ref|Homo sapiens acid phosphatase 5, tartrate resistant (ACP5), transcript variant 4, mRNA [NM_001611]	3,06
CHAF1B	NM_005441	ref|Homo sapiens chromatin assembly factor 1, subunit B (p60) (CHAF1B), mRNA [NM_005441]	3,06
AV749257	AV749257	gb|AV749257 AV749257 NPC Homo sapiens cDNA clone NPCCTB10 5', mRNA sequence [AV749257]	3,06
SPAG4	NM_003116	ref|Homo sapiens sperm associated antigen 4 (SPAG4), mRNA [NM_003116]	3,05
TM4SF19	NM_138461	ref|Homo sapiens transmembrane 4 L six family member 19 (TM4SF19), mRNA [NM_138461]	3,04
BAAT	NM_001701	ref|Homo sapiens bile acid Coenzyme A: amino acid N-acyltransferase (glycine N-choloyltransferase) (BAAT), transcript variant 1, mRNA [NM_001701]	3,04
CHAF1B	NM_005441	ref|Homo sapiens chromatin assembly factor 1, subunit B (p60) (CHAF1B), mRNA [NM_005441]	3,04
PELI3	NM_145065	ref|Homo sapiens pellino homolog 3 (Drosophila) (PELI3), transcript variant 1, mRNA [NM_145065]	3,04
TPK1	NM_022445	ref|Homo sapiens thiamin pyrophosphokinase 1 (TPK1), transcript variant 1, mRNA [NM_022445]	3,03
FCGR2A	NM_021642	ref|Homo sapiens Fc fragment of IgG, low affinity IIa, receptor (CD32) (FCGR2A), transcript variant 2, mRNA [NM_021642]	3,03
CHAF1B	NM_005441	ref|Homo sapiens chromatin assembly factor 1, subunit B (p60) (CHAF1B), mRNA [NM_005441]	3,03
FBLIM1	NM_017556	ref|Homo sapiens filamin binding LIM protein 1 (FBLIM1), transcript variant 1, mRNA [NM_017556]	3,02
CHAF1B	NM_005441	ref|Homo sapiens chromatin assembly factor 1, subunit B (p60) (CHAF1B), mRNA [NM_005441]	3,02
C10orf59	NM_001031709	ref|Homo sapiens chromosome 10 open reading frame 59 (C10orf59), transcript variant 1, mRNA [NM_001031709]	3,02
SPDYA	NM_001008779	ref|Homo sapiens speedy homolog A (Xenopus laevis) (SPDYA), transcript variant 2, mRNA [NM_001008779]	3,02
AK055428	AK055428	gb|Homo sapiens cDNA FLJ30866 fis, clone FEBRA2004110, highly similar to PHOSPHOLIPASE ADRAB-B PRECURSOR (EC 3.1.-.-). [AK055428]	3,02
PDPR	NM_017990	ref|Homo sapiens pyruvate dehydrogenase phosphatase regulatory subunit (PDPR), mRNA [NM_017990]	2,99
LOC388588	NR_024432	ref|Homo sapiens hypothetical LOC388588 (LOC388588), non-coding RNA [NR_024432]	2,99
IMMP2L	NM_032549	ref|Homo sapiens IMP2 inner mitochondrial membrane peptidase-like (S. cerevisiae) (IMMP2L), nuclear gene encoding mitochondrial protein, mRNA [NM_0325(	2,99
CHAF1B	NM_005441	ref|Homo sapiens chromatin assembly factor 1, subunit B (p60) (CHAF1B), mRNA [NM_005441]	2,99
MET	NM_000245	ref|Homo sapiens met proto-oncogene (hepatocyte growth factor receptor) (MET), transcript variant 2, mRNA [NM_000245]	2,98
BC008001	BC008001	gb|Homo sapiens mRNA similar to hypothetical protein FLJ21463 (cDNA clone IMAGE:3504595). [BC008001]	2,98
FCGR2B	NM_004001	ref|Homo sapiens Fc fragment of IgG, low affinity IIb, receptor (CD32) (FCGR2B), transcript variant 1, mRNA [NM_004001]	2,98
CHAF1B	NM_005441	ref|Homo sapiens chromatin assembly factor 1, subunit B (p60) (CHAF1B), mRNA [NM_005441]	2,97
MARCH3	NM_178450	ref|Homo sapiens membrane-associated ring finger (C3HC4) 3 (MARCH3), mRNA [NM_178450]	2,97
THC2533854	THC2533854	thc|Q2NUU5_SODGM (Q2NUU5) Glutamate-aspartate ABC transporter ATP-binding component GltL, partial (7%) [THC2533854]	2,97
LAMP3	NM_014398	ref|Homo sapiens lysosomal-associated membrane protein 3 (LAMP3), mRNA [NM_014398]	2,95
NDRG4	NM_022910	ref|Homo sapiens NDRG family member 4 (NDRG4), transcript variant 3, mRNA [NM_022910]	2,95
HIST1H4L	NM_003546	ref|Homo sapiens histone cluster 1, H4l (HIST1H4L), mRNA [NM_003546]	2,95
ADAM23	NM_003812	ref|Homo sapiens ADAM metallopeptidase domain 23 (ADAM23), mRNA [NM_003812]	2,94
LIMCH1	NM_014988	ref|Homo sapiens LIM and calponin homology domains 1 (LIMCH1), transcript variant 1, mRNA [NM_014988]	2,94
NAV2	NM_182964	ref|Homo sapiens neuron navigator 2 (NAV2), transcript variant 1, mRNA [NM_182964]	2,93
TLR4	NM_138554	ref|Homo sapiens toll-like receptor 4 (TLR4), transcript variant 1, mRNA [NM_138554]	2,93
ENST00000372142	ENST00000372142	ens|Pro-neuregulin-3, membrane-bound isoform Precursor (Pro-NRG3) [Contains Neuregulin-3(NRG-3)] [Source:UniProtKB/Swiss-Prot;Acc:P56975] [ENST000(	2,92
AB362555	AB362555	gb|Homo sapiens HLA-DMB mRNA for MHC class II antigen, partial cds, splicing variant 2. [AB362555]	2,91
CHAF1B	NM_005441	ref|Homo sapiens chromatin assembly factor 1, subunit B (p60) (CHAF1B), mRNA [NM_005441]	2,90

TTYH2	NM_032646	ref|Homo sapiens tweety homolog 2 (Drosophila) (TTYH2), transcript variant 1, mRNA [NM_032646]	2,90
TP53TG3	NM_016212	ref|Homo sapiens TP53 target 3 (TP53TG3), mRNA [NM_016212]	2,90
HIST1H4C	NM_003542	ref|Homo sapiens histone cluster 1, H4c (HIST1H4C), mRNA [NM_003542]	2,89
SNCB	NM_001001502	ref|Homo sapiens synuclein, beta (SNCB), transcript variant 1, mRNA [NM_001001502]	2,88
BCL2A1	NM_004049	ref|Homo sapiens BCL2-related protein A1 (BCL2A1), transcript variant 1, mRNA [NM_004049]	2,87
C9orf91	NM_153045	ref|Homo sapiens chromosome 9 open reading frame 91 (C9orf91), mRNA [NM_153045]	2,86
ESPN	NM_031475	ref|Homo sapiens espin (ESPN), mRNA [NM_031475]	2,86
GULP1	NM_016315	ref|Homo sapiens GULP, engulfment adaptor PTB domain containing 1 (GULP1), mRNA [NM_016315]	2,86
ZNF778	NM_182531	ref|Homo sapiens zinc finger protein 778 (ZNF778), mRNA [NM_182531]	2,86
ECOP	NM_030796	ref|Homo sapiens EGFR-coamplified and overexpressed protein (ECOP), mRNA [NM_030796]	2,85
RFX6	NM_173560	ref|Homo sapiens regulatory factor X, 6 (RFX6), mRNA [NM_173560]	2,84
DENND2D	AL713773	Unknown	2,83
MYB	NM_005375	ref|Homo sapiens v-myb myeloblastosis viral oncogene homolog (avian) (MYB), transcript variant 2, mRNA [NM_005375]	2,82
A_24_P127063	A_24_P127063	Unknown	2,81
CREB3L2	BC063666	gb|Homo sapiens cAMP responsive element binding protein 3-like 2, mRNA (cDNA clone IMAGE:4185677), complete cds. [BC063666]	2,79
DQ786230	DQ786230	gb|Homo sapiens clone HLS_IMAGE_1706664 mRNA sequence. [DQ786230]	2,77
RASSF4	NM_032023	ref|Homo sapiens Ras association (RalGDS/AF-6) domain family member 4 (RASSF4), mRNA [NM_032023]	2,77
GRAP	NM_006613	ref|Homo sapiens GRB2-related adaptor protein (GRAP), mRNA [NM_006613]	2,77
FGGY	NM_018291	ref|Homo sapiens FGGY carbohydrate kinase domain containing (FGGY), transcript variant 2, mRNA [NM_018291]	2,75
STARD8	NM_014725	ref|Homo sapiens StAR-related lipid transfer (START) domain containing 8 (STARD8), transcript variant 2, mRNA [NM_014725]	2,75
SNN	NM_003498	ref|Homo sapiens stannin (SNN), mRNA [NM_003498]	2,75
TRPM6	NM_017662	ref|Homo sapiens transient receptor potential cation channel, subfamily M, member 6 (TRPM6), mRNA [NM_017662]	2,74
GLB1L3	NM_001080407	ref|Homo sapiens galactosidase, beta 1-like 3 (GLB1L3), mRNA [NM_001080407]	2,74
PXMP2	NM_018663	ref|Homo sapiens peroxisomal membrane protein 2, 22kDa (PXMP2), mRNA [NM_018663]	2,73
SLC37A2	NM_198277	ref|Homo sapiens solute carrier family 37 (glycerol-3-phosphate transporter), member 2 (SLC37A2), mRNA [NM_198277]	2,72
CCR7	NM_001838	ref|Homo sapiens chemokine (C-C motif) receptor 7 (CCR7), mRNA [NM_001838]	2,71
A_32_P93792	A_32_P93792	Unknown	2,71
OLFM1	NM_006334	ref|Homo sapiens olfactomedin 1 (OLFM1), transcript variant 2, mRNA [NM_006334]	2,70
CACNA1G	NM_018896	ref|Homo sapiens calcium channel, voltage-dependent, T type, alpha 1G subunit (CACNA1G), transcript variant 1, mRNA [NM_018896]	2,70
CHCHD7	NM_001011667	ref|Homo sapiens coiled-coil-helix-coiled-coil-helix domain containing 7 (CHCHD7), transcript variant 1, mRNA [NM_001011667]	2,70
C5orf47	ENST00000340147	ens|Uncharacterized protein C5orf47 [Source:UniProtKB/Swiss-Prot;Acc:Q569G3] [ENST00000340147]	2,70
SLC5A4	NM_014227	ref|Homo sapiens solute carrier family 5 (low affinity glucose cotransporter), member 4 (SLC5A4), mRNA [NM_014227]	2,70
TMCC3	NM_020698	ref|Homo sapiens transmembrane and coiled-coil domain family 3 (TMCC3), mRNA [NM_020698]	2,70
THC2750781	THC2750781	Unknown	2,69
CTSA	NM_000308	ref|Homo sapiens cathepsin A (CTSA), transcript variant 1, mRNA [NM_000308]	2,68
ACN9	NM_020186	ref|Homo sapiens ACN9 homolog (S. cerevisiae) (ACN9), mRNA [NM_020186]	2,65
WISP2	NM_003881	ref|Homo sapiens WNT1 inducible signaling pathway protein 2 (WISP2), mRNA [NM_003881]	2,65
DHRS13	NM_144683	ref|Homo sapiens dehydrogenase/reductase (SDR family) member 13 (DHRS13), mRNA [NM_144683]	2,65
TP53TG3	NM_016212	ref|Homo sapiens TP53 target 3 (TP53TG3), mRNA [NM_016212]	2,64
OXNAD1	NM_138381	ref|Homo sapiens oxidoreductase NAD-binding domain containing 1 (OXNAD1), mRNA [NM_138381]	2,63
BC015977	BC015977	gb|Homo sapiens, clone IMAGE:4042121, mRNA, partial cds. [BC015977]	2,61
MGC16025	BC008026	gb|Homo sapiens hypothetical protein MGC16025, mRNA (cDNA clone MGC:16025 IMAGE:3607793), complete cds. [BC008026]	2,61
RAD51L1	NM_133510	ref|Homo sapiens RAD51-like 1 (S. cerevisiae) (RAD51L1), transcript variant 2, mRNA [NM_133510]	2,58
RBM33	BC011923	gb|Homo sapiens RNA binding motif protein 33, mRNA (cDNA clone IMAGE:4542314), complete cds. [BC011923]	2,52

Supplementary Table 2. Known MITF target genes dowregulated more than 2-fold by Brg1 knockdown in 501mel cells (P<0.005). (MITF itself was downregulated ~5-fold).

Gene symbol	Gene name	Genebank	-fold
DCT	dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2) (DCT),transcript variant 1, mRNA	NM_001922	22.50
TRPM1	transient receptor potential cation channel, subfamily M, member 1 (TRPM1), mRNA	NM_002420	15.98
MLANA	melan-A (MLANA), mRNA	NM_005511	10.76
RAB27A	RAB27A, member RAS oncogene family (RAB27A), transcript variant 1, mRNA	NM_004580	6.72
BIRC7	baculoviral IAP repeat-containing 7 (BIRC7), transcript variant 2, mRNA	NM_022161	5.85
CDK2	cyclin-dependent kinase 2 (CDK2), transcript variant 1, mRNA	NM_001798	4.98
TYRP1	tyrosinase-related protein 1 (TYRP1), mRNA	NM_000550	4.98
SILV	silver homolog (mouse) (SILV), mRNA	NM_006928	4.21
SLC45A2	solute carrier family 45, member 2 (SLC45A2), transcript variant 1, mRNA	NM_016180	4.12
SNAI2	snail homolog 2 (Drosophila) (SNAI2), mRNA	NM_003068	3,76
TYR	tyrosinase (oculocutaneous albinism IA) (TYR), mRNA	NM_000372	3.49
GPR143	G protein-coupled receptor 143 (GPR143), mRNA	NM_000273	3.27
MET	met proto-oncogene (hepatocyte growth factor receptor) (MET), transcript variant 2, mRNA	NM_000245	2.98
BCL2	B-cell CLL/lymphoma 2 (BCL2), nuclear gene encoding mitochondrial protein, transcript variant alpha, mRNA	NM_000633	2.38
EDNRB	endothelin receptor type B (EDNRB), transcript variant 2, mRNA	NM_003991	2.23

Supplementary Table S3. Microarray analysis of genes up-regulated >2-fold (P<0.005) by Brg1 depletion in 501mel cells.

Gene Symbol	Systematic Name	Gene Title	Fold change
COL13A1	NM_005203	ref|Homo sapiens collagen, type XIII, alpha 1 (COL13A1), transcript variant 1, mRNA [NM_005203]	23,203
COL13A1	NM_005203	ref|Homo sapiens collagen, type XIII, alpha 1 (COL13A1), transcript variant 1, mRNA [NM_005203]	20,793
COL13A1	NM_005203	ref|Homo sapiens collagen, type XIII, alpha 1 (COL13A1), transcript variant 1, mRNA [NM_005203]	20,769
COL13A1	NM_005203	ref|Homo sapiens collagen, type XIII, alpha 1 (COL13A1), transcript variant 1, mRNA [NM_005203]	19,676
COL13A1	NM_005203	ref|Homo sapiens collagen, type XIII, alpha 1 (COL13A1), transcript variant 1, mRNA [NM_005203]	19,074
NPTX1	NM_002522	ref|Homo sapiens neuronal pentraxin I (NPTX1), mRNA [NM_002522]	18,548
COL13A1	NM_005203	ref|Homo sapiens collagen, type XIII, alpha 1 (COL13A1), transcript variant 1, mRNA [NM_005203]	18,126
HMGCS1	NM_002130	ref|Homo sapiens 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble) (HMGCS1), transcript variant 2, mRNA [NM_002	15,489
COL13A1	NM_005203	ref|Homo sapiens collagen, type XIII, alpha 1 (COL13A1), transcript variant 1, mRNA [NM_005203]	14,214
COL13A1	NM_005203	ref|Homo sapiens collagen, type XIII, alpha 1 (COL13A1), transcript variant 1, mRNA [NM_005203]	14,073
CXCL14	NM_004887	ref|Homo sapiens chemokine (C-X-C motif) ligand 14 (CXCL14), mRNA [NM_004887]	13,918
COL13A1	NM_005203	ref|Homo sapiens collagen, type XIII, alpha 1 (COL13A1), transcript variant 1, mRNA [NM_005203]	12,630
COL13A1	NM_005203	ref|Homo sapiens collagen, type XIII, alpha 1 (COL13A1), transcript variant 1, mRNA [NM_005203]	11,752
KRT34	NM_021013	ref|Homo sapiens keratin 34 (KRT34), mRNA [NM_021013]	11,283
COL13A1	NM_005203	ref|Homo sapiens collagen, type XIII, alpha 1 (COL13A1), transcript variant 1, mRNA [NM_005203]	10,367
HMGCS1	NM_002130	ref|Homo sapiens 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble) (HMGCS1), transcript variant 2, mRNA [NM_002	10,278
ITGA1	NM_181501	ref|Homo sapiens integrin, alpha 1 (ITGA1), mRNA [NM_181501]	10,271
KCNN4	NM_002250	ref|Homo sapiens potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4 (KCNN4), mRN	9,800
THC2681410	THC2681410	Unknown	9,550
HIST2H2AA4	NM_001040874	ref|Homo sapiens histone cluster 2, H2aa4 (HIST2H2AA4), mRNA [NM_001040874]	9,185
HIST2H2AA4	NM_001040874	ref|Homo sapiens histone cluster 2, H2aa4 (HIST2H2AA4), mRNA [NM_001040874]	8,980
HSD17B7P2	AF165514	gb|Homo sapiens 17-beta-hydroxysteroid dehydrogenase type VII isoform mRNA, complete cds. [AF165514]	8,211
ACAT2	NM_005891	ref|Homo sapiens acetyl-Coenzyme A acetyltransferase 2 (ACAT2), mRNA [NM_005891]	8,175
A_23_P147404	A_23_P147404	Unknown	7,967
ACAT2	NM_005891	ref|Homo sapiens acetyl-Coenzyme A acetyltransferase 2 (ACAT2), mRNA [NM_005891]	7,966
LSS	NM_001001438	ref|Homo sapiens lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase) (LSS), transcript variant 2, mRNA [NM_001001438	7,672
NPTX1	NM_002522	ref|Homo sapiens neuronal pentraxin I (NPTX1), mRNA [NM_002522]	7,658
HSD17B7	NM_016371	ref|Homo sapiens hydroxysteroid (17-beta) dehydrogenase 7 (HSD17B7), mRNA [NM_016371]	7,635
HSD17B7	NM_016371	ref|Homo sapiens hydroxysteroid (17-beta) dehydrogenase 7 (HSD17B7), mRNA [NM_016371]	7,312
IDI1	NM_004508	ref|Homo sapiens isopentenyl-diphosphate delta isomerase 1 (IDI1), mRNA [NM_004508]	7,103
MST150	NM_032947	ref|Homo sapiens MSTP150 (MST150), mRNA [NM_032947]	7,028
DKK1	NM_012242	ref|Homo sapiens dickkopf homolog 1 (Xenopus laevis) (DKK1), mRNA [NM_012242]	7,023
HIST1H2AD	NM_021065	ref|Homo sapiens histone cluster 1, H2ad (HIST1H2AD), mRNA [NM_021065]	6,921
TMEM158	NM_015444	ref|Homo sapiens transmembrane protein 158 (TMEM158), mRNA [NM_015444]	6,886
LDLR	NM_000527	ref|Homo sapiens low density lipoprotein receptor (LDLR), mRNA [NM_000527]	6,476
IL3RA	NM_002183	ref|Homo sapiens interleukin 3 receptor, alpha (low affinity) (IL3RA), mRNA [NM_002183]	6,409
IL21R	NM_181078	ref|Homo sapiens interleukin 21 receptor (IL21R), transcript variant 2, mRNA [NM_181078]	6,344
LSS	NM_001001438	ref|Homo sapiens lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase) (LSS), transcript variant 2, mRNA [NM_001001438	6,340
HIST1H1C	NM_005319	ref|Homo sapiens histone cluster 1, H1c (HIST1H1C), mRNA [NM_005319]	6,301
EGR4	NM_001965	ref|Homo sapiens early growth response 4 (EGR4), mRNA [NM_001965]	6,293
HIST2H2AB	NM_175065	ref|Homo sapiens histone cluster 2, H2ab (HIST2H2AB), mRNA [NM_175065]	6,230
LRRN3	NM_018334	ref|Homo sapiens leucine rich repeat neuronal 3 (LRRN3), transcript variant 3, mRNA [NM_018334]	6,210
SC4MOL	NM_006745	ref|Homo sapiens sterol-C4-methyl oxidase-like (SC4MOL), transcript variant 1, mRNA [NM_006745]	6,173
LDLR	NM_000527	ref|Homo sapiens low density lipoprotein receptor (LDLR), mRNA [NM_000527]	6,126
CADPS	NM_183393	ref|Homo sapiens Ca++-dependent secretion activator (CADPS), transcript variant 3, mRNA [NM_183393]	6,054
CDKN2B	NM_078487	ref|Homo sapiens cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) (CDKN2B), transcript variant 2, mRNA [NM_078487]	6,004
NSDHL	NM_015922	ref|Homo sapiens NAD(P) dependent steroid dehydrogenase-like (NSDHL), transcript variant 1, mRNA [NM_015922]	5,936
AW993939	AW993939	gb|AW993939 RC1-BN0035-130400-013-b06 BN0035 Homo sapiens cDNA, mRNA sequence [AW993939]	5,921
CR603951	CR603951	gb|full-length cDNA clone CS0DM011YC22 of Fetal liver of Homo sapiens (human). [CR603951]	5,865
FDPS	NM_002004	ref|Homo sapiens farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyltranstransferase, geranyltrans	5,800
UNC5B	NM_170744	ref|Homo sapiens unc-5 homolog B (C. elegans) (UNC5B), mRNA [NM_170744]	5,763
PPFIBP1	NM_003622	ref|Homo sapiens PTPRF interacting protein, binding protein 1 (liprin beta 1) (PPFIBP1), transcript variant 1, mRNA [NM_00362	5,756
A_23_P170713	A_23_P170713	Unknown	5,751
ACSS2	NM_018677	ref|Homo sapiens acyl-CoA synthetase short-chain family member 2 (ACSS2), transcript variant 1, mRNA [NM_018677]	5,729
HIST1H2AE	NM_021052	ref|Homo sapiens histone cluster 1, H2ae (HIST1H2AE), mRNA [NM_021052]	5,681
ADAM19	NM_033274	ref|Homo sapiens ADAM metallopeptidase domain 19 (meltrin beta) (ADAM19), transcript variant 2, mRNA [NM_033274]	5,681
GZMA	NM_006144	ref|Homo sapiens granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3) (GZMA), mRNA [NM_006144]	5,661
SLC1A3	NM_004172	ref|Homo sapiens solute carrier family 1 (glial high affinity glutamate transporter), member 3 (SLC1A3), mRNA [NM_004172]	5,641
X15674	X15674	gb|Human pTR5 mRNA for repetitive sequence. [X15674]	5,571
FDPSL2A	NR_003262	ref|Homo sapiens MGC44478 (FDPSL2A), non-coding RNA [NR_003262]	5,566
PPFIBP1	NM_003622	ref|Homo sapiens PTPRF interacting protein, binding protein 1 (liprin beta 1) (PPFIBP1), transcript variant 1, mRNA [NM_00362	5,518
INSIG1	NM_198336	ref|Homo sapiens insulin induced gene 1 (INSIG1), transcript variant 2, mRNA [NM_198336]	5,457
MVK	NM_000431	ref|Homo sapiens mevalonate kinase (MVK), transcript variant 1, mRNA [NM_000431]	5,366
VWCE	NM_152718	ref|Homo sapiens von Willebrand factor C and EGF domains (VWCE), mRNA [NM_152718]	5,356
HIST2H2AC	NM_003517	ref|Homo sapiens histone cluster 2, H2ac (HIST2H2AC), mRNA [NM_003517]	5,303
PPFIBP1	NM_003622	ref|Homo sapiens PTPRF interacting protein, binding protein 1 (liprin beta 1) (PPFIBP1), transcript variant 1, mRNA [NM_00362	5,295
PMEPA1	NM_020182	ref|Homo sapiens prostate transmembrane protein, androgen induced 1 (PMEPA1), transcript variant 1, mRNA [NM_020182]	5,225
A_24_P67268	A_24_P67268	Unknown	5,225
THC2523793	THC2523793	thc|Q9DC07_MOUSE (Q9DC07) Adult male lung cDNA, RIKEN full-length enriched library, clone:1200007O21 product:LIM AND	5,216
BMP6	NM_001718	ref|Homo sapiens bone morphogenetic protein 6 (BMP6), mRNA [NM_001718]	5,082
TGFBI	NM_000358	ref|Homo sapiens transforming growth factor, beta-induced, 68kDa (TGFBI), mRNA [NM_000358]	5,081
ACSS2	NM_018677	ref|Homo sapiens acyl-CoA synthetase short-chain family member 2 (ACSS2), transcript variant 1, mRNA [NM_018677]	5,055
ANKRD36B	AK024934	gb|Homo sapiens cDNA: FLJ21281 fis, clone COL01904. [AK024934]	5,019
RIMS3	NM_014747	ref|Homo sapiens regulating synaptic membrane exocytosis 3 (RIMS3), mRNA [NM_014747]	5,010
CDKN2B	NM_004936	ref|Homo sapiens cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) (CDKN2B), transcript variant 1, mRNA [NM_004936]	4,996
DHCR7	NM_001360	ref|Homo sapiens 7-dehydrocholesterol reductase (DHCR7), mRNA [NM_001360]	4,985
CXCL1	NM_001511	ref|Homo sapiens chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) (CXCL1), mRNA [NM_001511	4,843
EIF4G3	NM_003760	ref|Homo sapiens eukaryotic translation initiation factor 4 gamma, 3 (EIF4G3), mRNA [NM_003760]	4,832
TMEM55B	NM_144568	ref|Homo sapiens transmembrane protein 55B (TMEM55B), transcript variant 2, mRNA [NM_144568]	4,810
IQCD	NM_138451	ref|Homo sapiens IQ motif containing D (IQCD), mRNA [NM_138451]	4,805
FDFT1	NM_004462	ref|Homo sapiens farnesyl-diphosphate farnesyltransferase 1 (FDFT1), mRNA [NM_004462]	4,688
PLAC2	BC036545	gb|Homo sapiens placenta-specific 2 (non-protein coding), mRNA (cDNA clone MGC:43133 IMAGE:5261222), complete cds. [B(	4,656
USP36	AK022913	gb|Homo sapiens cDNA FLJ12851 fis, clone NT2RP2003401, weakly similar to UBIQUITIN CARBOXYL-TERMINAL HYDROLAS	4,628
KIAA1683	NM_025249	ref|Homo sapiens KIAA1683 (KIAA1683), mRNA [NM_025249]	4,620
ATP2A1	NM_173201	ref|Homo sapiens ATPase, Ca++ transporting, cardiac muscle, fast twitch 1 (ATP2A1), transcript variant a, mRNA [NM_173201]	4,619
JAM2	NM_021219	ref|Homo sapiens junctional adhesion molecule 2 (JAM2), mRNA [NM_021219]	4,608
WDR37	NM_014023	ref|Homo sapiens WD repeat domain 37 (WDR37), mRNA [NM_014023]	4,580
CYR61	NM_001554	ref|Homo sapiens cysteine-rich, angiogenic inducer, 61 (CYR61), mRNA [NM_001554]	4,578
HMGCR	NM_000859	ref|Homo sapiens 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR), transcript variant 1, mRNA [NM_000859]	4,562
DOCK4	NM_014705	ref|Homo sapiens dedicator of cytokinesis 4 (DOCK4), mRNA [NM_014705]	4,554
ANKRD23	NM_144994	ref|Homo sapiens ankyrin repeat domain 23 (ANKRD23), mRNA [NM_144994]	4,512
C14orf1	NM_007176	ref|Homo sapiens chromosome 14 open reading frame 1 (C14orf1), mRNA [NM_007176]	4,484
FAM105A	NM_019018	ref|Homo sapiens family with sequence similarity 105, member A (FAM105A), mRNA [NM_019018]	4,457
MVK	NM_000431	ref|Homo sapiens mevalonate kinase (MVK), transcript variant 1, mRNA [NM_000431]	4,452
A_24_P834646	A_24_P834646	Unknown	4,450
SQLE	NM_003129	ref|Homo sapiens squalene epoxidase (SQLE), mRNA [NM_003129]	4,449
MVD	NM_002461	ref|Homo sapiens mevalonate (diphospho) decarboxylase (MVD), mRNA [NM_002461]	4,423
LMO2	NM_005574	ref|Homo sapiens LIM domain only 2 (rhombotin-like 1) (LMO2), transcript variant 1, mRNA [NM_005574]	4,357
ASGR1	NM_001671	ref|Homo sapiens asialoglycoprotein receptor 1 (ASGR1), mRNA [NM_001671]	4,330
LMO2	NM_005574	ref|Homo sapiens LIM domain only 2 (rhombotin-like 1) (LMO2), transcript variant 1, mRNA [NM_005574]	4,325
IL22RA1	NM_021258	ref|Homo sapiens interleukin 22 receptor, alpha 1 (IL22RA1), mRNA [NM_021258]	4,274
PEG10	NM_001040152	ref|Homo sapiens paternally expressed 10 (PEG10), transcript variant 1, mRNA [NM_001040152]	4,257

MGC23284	NR_024399	ref|Homo sapiens hypothetical LOC197187 (MGC23284), transcript variant 2, non-coding RNA [NR_024399]	4,252
DERL3	NM_198440	ref|Homo sapiens Der1-like domain family, member 3 (DERL3), transcript variant 3, mRNA [NM_198440]	4,215
THC2655811	THC2655811	Unknown	4,166
PRDM2	NM_012231	ref|Homo sapiens PR domain containing 2, with ZNF domain (PRDM2), transcript variant 1, mRNA [NM_012231]	4,164
ITPKA	NM_002220	ref|Homo sapiens inositol 1,4,5-trisphosphate 3-kinase A (ITPKA), mRNA [NM_002220]	4,132
CYR61	NM_001554	ref|Homo sapiens cysteine-rich, angiogenic inducer, 61 (CYR61), mRNA [NM_001554]	4,115
WNT11	NM_004626	ref|Homo sapiens wingless-type MMTV integration site family, member 11 (WNT11), mRNA [NM_004626]	4,096
UNC5CL	NM_173561	ref|Homo sapiens unc-5 homolog C (C. elegans)-like (UNC5CL), mRNA [NM_173561]	4,065
CYP51A1	NM_000786	ref|Homo sapiens cytochrome P450, family 51, subfamily A, polypeptide 1 (CYP51A1), mRNA [NM_000786]	4,057
PIK3IP1	NM_052880	ref|Homo sapiens phosphoinositide-3-kinase interacting protein 1 (PIK3IP1), transcript variant 1, mRNA [NM_052880]	4,009
IGDCC3	NM_004884	ref|Homo sapiens immunoglobulin superfamily, DCC subclass, member 3 (IGDCC3), mRNA [NM_004884]	3,990
HIST2H2BE	NM_003528	ref|Homo sapiens histone cluster 2, H2be (HIST2H2BE), mRNA [NM_003528]	3,982
CYB5B	NM_030579	ref|Homo sapiens cytochrome b5 type B (outer mitochondrial membrane) (CYB5B), nuclear gene encoding mitochondrial protein	3,949
ZNF720	NM_001130913	ref|Homo sapiens zinc finger protein 720 (ZNF720), mRNA [NM_001130913]	3,924
HIST1H1D	NM_005320	ref|Homo sapiens histone cluster 1, H1d (HIST1H1D), mRNA [NM_005320]	3,905
MMAB	NM_052845	ref|Homo sapiens methylmalonic aciduria (cobalamin deficiency) cblB type (MMAB), nuclear gene encoding mitochondrial proteir	3,890
LPIN1	NM_145693	ref|Homo sapiens lipin 1 (LPIN1), mRNA [NM_145693]	3,873
SCARA5	NM_173833	ref|Homo sapiens scavenger receptor class A, member 5 (putative) (SCARA5), mRNA [NM_173833]	3,867
TM7SF2	NM_003273	ref|Homo sapiens transmembrane 7 superfamily member 2 (TM7SF2), mRNA [NM_003273]	3,862
OVGP1	NM_002557	ref|Homo sapiens oviductal glycoprotein 1, 120kDa (OVGP1), mRNA [NM_002557]	3,815
SERPINB8	NM_001031848	ref|Homo sapiens serpin peptidase inhibitor, clade B (ovalbumin), member 8 (SERPINB8), transcript variant 3, mRNA [NM_0010	3,812
LMO2	NM_005574	ref|Homo sapiens LIM domain only 2 (rhombotin-like 1) (LMO2), transcript variant 1, mRNA [NM_005574]	3,807
LMO2	NM_005574	ref|Homo sapiens LIM domain only 2 (rhombotin-like 1) (LMO2), transcript variant 1, mRNA [NM_005574]	3,807
BDNF	NM_170735	ref|Homo sapiens brain-derived neurotrophic factor (BDNF), transcript variant 1, mRNA [NM_170735]	3,796
TM9SF4	NM_014742	ref|Homo sapiens transmembrane 9 superfamily protein member 4 (TM9SF4), mRNA [NM_014742]	3,788
TESC	NM_017899	ref|Homo sapiens tescalcin (TESC), mRNA [NM_017899]	3,779
A_24_P196134	A_24_P196134	Unknown	3,777
CYP51A1	NM_000786	ref|Homo sapiens cytochrome P450, family 51, subfamily A, polypeptide 1 (CYP51A1), mRNA [NM_000786]	3,762
JUN	NM_002228	ref|Homo sapiens jun oncogene (JUN), mRNA [NM_002228]	3,762
CYR61	NM_001554	ref|Homo sapiens cysteine-rich, angiogenic inducer, 61 (CYR61), mRNA [NM_001554]	3,754
SSTR2	NM_001050	ref|Homo sapiens somatostatin receptor 2 (SSTR2), mRNA [NM_001050]	3,750
JUN	NM_002228	ref|Homo sapiens jun oncogene (JUN), mRNA [NM_002228]	3,744
PTGS2	NM_000963	ref|Homo sapiens prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), mRNA [N	3,741
DNAJB6	NM_058246	ref|Homo sapiens DnaJ (Hsp40) homolog, subfamily B, member 6 (DNAJB6), transcript variant 1, mRNA [NM_058246]	3,732
MMAB	NM_052845	ref|Homo sapiens methylmalonic aciduria (cobalamin deficiency) cblB type (MMAB), nuclear gene encoding mitochondrial proteir	3,729
JUN	NM_002228	ref|Homo sapiens jun oncogene (JUN), mRNA [NM_002228]	3,722
THC2665398	THC2665398	thc|Q5T0S6_HUMAN (Q5T0S6) Palmitoyl-protein thioesterase 1 (Ceroid-lipofuscinosis, neuronal 1, infantile) (Fragment), partial	3,705
SYNPO2	NM_001128933	ref|Homo sapiens synaptopodin 2 (SYNPO2), transcript variant 2, mRNA [NM_001128933]	3,703
TDO2	NM_005651	ref|Homo sapiens tryptophan 2,3-dioxygenase (TDO2), mRNA [NM_005651]	3,700
ARMC9	NM_025139	ref|Homo sapiens armadillo repeat containing 9 (ARMC9), mRNA [NM_025139]	3,699
LOC150759	AK057596	gb|Homo sapiens cDNA FLJ33034 fis, clone THYMU2000236. [AK057596]	3,660
PTGS2	NM_000963	ref|Homo sapiens prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), mRNA [N	3,656
YPEL2	NM_001005404	ref|Homo sapiens yippee-like 2 (Drosophila) (YPEL2), mRNA [NM_001005404]	3,654
JUN	NM_002228	ref|Homo sapiens jun oncogene (JUN), mRNA [NM_002228]	3,628
JUN	NM_002228	ref|Homo sapiens jun oncogene (JUN), mRNA [NM_002228]	3,613
LMO2	NM_005574	ref|Homo sapiens LIM domain only 2 (rhombotin-like 1) (LMO2), transcript variant 1, mRNA [NM_005574]	3,589
SLC35F1	NM_001029858	ref|Homo sapiens solute carrier family 35, member F1 (SLC35F1), mRNA [NM_001029858]	3,586
NR2F1	NM_005654	ref|Homo sapiens nuclear receptor subfamily 2, group F, member 1 (NR2F1), mRNA [NM_005654]	3,578
THC2577566	THC2577566	Unknown	3,572
JUN	NM_002228	ref|Homo sapiens jun oncogene (JUN), mRNA [NM_002228]	3,560
PPFIBP1	NM_177444	ref|Homo sapiens PTPRF interacting protein, binding protein 1 (liprin beta 1) (PPFIBP1), transcript variant 2, mRNA [NM_177444	3,530
PTGS2	NM_000963	ref|Homo sapiens prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), mRNA [N	3,518
JUN	NM_002228	ref|Homo sapiens jun oncogene (JUN), mRNA [NM_002228]	3,517
JUN	NM_002228	ref|Homo sapiens jun oncogene (JUN), mRNA [NM_002228]	3,502
WFIKKN1	NM_053284	ref|Homo sapiens WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 1 (WFIKKN1), mRNA [NM_053284	3,486
JUN	NM_002228	ref|Homo sapiens jun oncogene (JUN), mRNA [NM_002228]	3,478
JUN	NM_002228	ref|Homo sapiens jun oncogene (JUN), mRNA [NM_002228]	3,463
ZNF425	NM_001001661	ref|Homo sapiens zinc finger protein 425 (ZNF425), mRNA [NM_001001661]	3,461
HBP1	NM_012257	ref|Homo sapiens HMG-box transcription factor 1 (HBP1), mRNA [NM_012257]	3,461
MAP2	NM_002374	ref|Homo sapiens microtubule-associated protein 2 (MAP2), transcript variant 1, mRNA [NM_002374]	3,461
BASP1	NM_006317	ref|Homo sapiens brain abundant, membrane attached signal protein 1 (BASP1), mRNA [NM_006317]	3,459
AF087999	AF087999	gb|Homo sapiens full length insert cDNA clone YX44E03. [AF087999]	3,452
GPR35	NM_005301	ref|Homo sapiens G protein-coupled receptor 35 (GPR35), mRNA [NM_005301]	3,451
ZNF75D	NM_007131	ref|Homo sapiens zinc finger protein 75D (ZNF75D), mRNA [NM_007131]	3,434
AOC3	NM_003734	ref|Homo sapiens amine oxidase, copper containing 3 (vascular adhesion protein 1) (AOC3), mRNA [NM_003734]	3,424
ZNF264	NM_003417	ref|Homo sapiens zinc finger protein 264 (ZNF264), mRNA [NM_003417]	3,417
TMEM97	NM_014573	ref|Homo sapiens transmembrane protein 97 (TMEM97), mRNA [NM_014573]	3,412
TP53	NM_000546	ref|Homo sapiens tumor protein p53 (TP53), transcript variant 1, mRNA [NM_000546]	3,403
TP53	NM_000546	ref|Homo sapiens tumor protein p53 (TP53), transcript variant 1, mRNA [NM_000546]	3,396
LMO2	NM_005574	ref|Homo sapiens LIM domain only 2 (rhombotin-like 1) (LMO2), transcript variant 1, mRNA [NM_005574]	3,393
LMO2	NM_005574	ref|Homo sapiens LIM domain only 2 (rhombotin-like 1) (LMO2), transcript variant 1, mRNA [NM_005574]	3,388
MXD1	NM_002357	ref|Homo sapiens MAX dimerization protein 1 (MXD1), mRNA [NM_002357]	3,365
YPEL2	NM_001005404	ref|Homo sapiens yippee-like 2 (Drosophila) (YPEL2), mRNA [NM_001005404]	3,351
CTSK	NM_000396	ref|Homo sapiens cathepsin K (CTSK), mRNA [NM_000396]	3,350
TMEM97	NM_014573	ref|Homo sapiens transmembrane protein 97 (TMEM97), mRNA [NM_014573]	3,337
TMEM97	NM_014573	ref|Homo sapiens transmembrane protein 97 (TMEM97), mRNA [NM_014573]	3,335
DOCK4	NM_014705	ref|Homo sapiens dedicator of cytokinesis 4 (DOCK4), mRNA [NM_014705]	3,333
RNF20	NM_019592	ref|Homo sapiens ring finger protein 20 (RNF20), mRNA [NM_019592]	3,324
HIST1H2AC	ENST00000314088	ens|Histone H2A type 1-C (H2A/I) [Source:UniProtKB/Swiss-Prot;Acc:Q93077] [ENST00000314088]	3,302
RDH16	NM_003708	ref|Homo sapiens retinol dehydrogenase 16 (all-trans) (RDH16), mRNA [NM_003708]	3,301
SC5DL	NM_001024956	ref|Homo sapiens sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, S. cerevisiae)-like (SC5DL), transcript variant 2, mR	3,292
GNG11	NM_004126	ref|Homo sapiens guanine nucleotide binding protein (G protein), gamma 11 (GNG11), mRNA [NM_004126]	3,290
PLAUR	NM_001005377	ref|Homo sapiens plasminogen activator, urokinase receptor (PLAUR), transcript variant 3, mRNA [NM_001005377]	3,273
GREM2	NM_022469	ref|Homo sapiens gremlin 2, cysteine knot superfamily, homolog (Xenopus laevis) (GREM2), mRNA [NM_022469]	3,273
PTGER4	NM_000958	ref|Homo sapiens prostaglandin E receptor 4 (subtype EP4) (PTGER4), mRNA [NM_000958]	3,271
IL8	NM_000584	ref|Homo sapiens interleukin 8 (IL8), mRNA [NM_000584]	3,264
LMO2	NM_005574	ref|Homo sapiens LIM domain only 2 (rhombotin-like 1) (LMO2), transcript variant 1, mRNA [NM_005574]	3,261
TMEM81	NM_203376	ref|Homo sapiens transmembrane protein 81 (TMEM81), mRNA [NM_203376]	3,253
FRG2	NM_001005217	ref|Homo sapiens FSHD region gene 2 (FRG2), mRNA [NM_001005217]	3,236
BX107298	BX107298	gb|BX107298 Soares_testis_NHT Homo sapiens cDNA clone IMAGp998N011789, mRNA sequence [BX107298]	3,227
TFPI	NM_006287	ref|Homo sapiens tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor) (TFPI), transcript variant 1, mRNA	3,183
ADAM8	NM_001109	ref|Homo sapiens ADAM metallopeptidase domain 8 (ADAM8), mRNA [NM_001109]	3,179
CR596670	CR596670	gb|full-length cDNA clone CS0DF018YM18 of Fetal brain of Homo sapiens (human). [CR596670]	3,173
CLCN6	NM_001286	ref|Homo sapiens chloride channel 6 (CLCN6), transcript variant CIC-6a, mRNA [NM_001286]	3,171
ERO1LB	NM_019891	ref|Homo sapiens ERO1-like beta (S. cerevisiae) (ERO1LB), mRNA [NM_019891]	3,150
TMEFF2	AB004064	gb|Homo sapiens mRNA for tomoregulin, complete cds. [AB004064]	3,149
SC5DL	NM_001024956	ref|Homo sapiens sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, S. cerevisiae)-like (SC5DL), transcript variant 2, mR	3,142
PTGS2	NM_000963	ref|Homo sapiens prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), mRNA [N	3,132
PDGFA	NM_002607	ref|Homo sapiens platelet-derived growth factor alpha polypeptide (PDGFA), transcript variant 1, mRNA [NM_002607]	3,112
HBP1	NM_012257	ref|Homo sapiens HMG-box transcription factor 1 (HBP1), mRNA [NM_012257]	3,110
C17orf59	NM_017622	ref|Homo sapiens chromosome 17 open reading frame 59 (C17orf59), mRNA [NM_017622]	3,105
MXD1	NM_002357	ref|Homo sapiens MAX dimerization protein 1 (MXD1), mRNA [NM_002357]	3,090
SCHIP1	NM_014575	ref|Homo sapiens schwannomin interacting protein 1 (SCHIP1), mRNA [NM_014575]	3,086
AA593970	AA593970	gb|AA593970 nn01c05.s1 NCI_CGAP_Co9 Homo sapiens cDNA clone IMAGE:1076456 3', mRNA sequence [AA593970]	3,073
FASN	NM_004104	ref|Homo sapiens fatty acid synthase (FASN), mRNA [NM_004104]	3,071

GOLGB1	NM_004487	ref|Homo sapiens golgin B1, golgi integral membrane protein (GOLGB1), mRNA [NM_004487]	3,057
TMEM49	BC024020	gb|Homo sapiens transmembrane protein 49, mRNA (cDNA clone IMAGE:4295896), with apparent retained intron. [BC024020]	3,056
PLA2G4C	NM_003706	ref|Homo sapiens phospholipase A2, group IVC (cytosolic, calcium-independent) (PLA2G4C), mRNA [NM_003706]	3,056
ENST00000298876	ENST00000298876	ens|Uncharacterized protein C12orf70 [Source:UniProtKB/Swiss-Prot;Acc:A6NFE2] [ENST00000298876]	3,048
GADD45A	NM_001924	ref|Homo sapiens growth arrest and DNA-damage-inducible, alpha (GADD45A), mRNA [NM_001924]	3,039
C4orf18	NM_016613	ref|Homo sapiens chromosome 4 open reading frame 18 (C4orf18), transcript variant 2, mRNA [NM_016613]	3,032
AMOTL2	NM_016201	ref|Homo sapiens angiomotin like 2 (AMOTL2), mRNA [NM_016201]	3,028
ADAMTS1	NM_006988	ref|Homo sapiens ADAM metallopeptidase with thrombospondin type 1 motif, 1 (ADAMTS1), mRNA [NM_006988]	3,021
CASP4	NM_033306	ref|Homo sapiens caspase 4, apoptosis-related cysteine peptidase (CASP4), transcript variant gamma, mRNA [NM_033306]	3,015
TM7SF2	NM_003273	ref|Homo sapiens transmembrane 7 superfamily member 2 (TM7SF2), mRNA [NM_003273]	3,012
NXN	NM_022463	ref|Homo sapiens nucleoredoxin (NXN), mRNA [NM_022463]	3,009
AK022044	AK022044	gb|Homo sapiens cDNA FLJ11982 fis, clone HEMBB1001335. [AK022044]	3,006
C17orf59	NM_017622	ref|Homo sapiens chromosome 17 open reading frame 59 (C17orf59), mRNA [NM_017622]	2,995
HOXB3	NM_002146	ref|Homo sapiens homeobox B3 (HOXB3), mRNA [NM_002146]	2,976
ABCB1	NM_000927	ref|Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 1 (ABCB1), mRNA [NM_000927]	2,975
ABCB1	NM_000927	ref|Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 1 (ABCB1), mRNA [NM_000927]	2,968
FAM132A	NM_001014980	ref|Homo sapiens family with sequence similarity 132, member A (FAM132A), mRNA [NM_001014980]	2,959
CUL4B	NM_003588	ref|Homo sapiens cullin 4B (CUL4B), transcript variant 1, mRNA [NM_003588]	2,955
GGPS1	NM_004837	ref|Homo sapiens geranylgeranyl diphosphate synthase 1 (GGPS1), transcript variant 1, mRNA [NM_004837]	2,952
ABCB1	NM_000927	ref|Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 1 (ABCB1), mRNA [NM_000927]	2,952
PTGS2	NM_000963	ref|Homo sapiens prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), mRNA [N	2,944
ERO1LB	NM_019891	ref|Homo sapiens ERO1-like beta (S. cerevisiae) (ERO1LB), mRNA [NM_019891]	2,939
THC2755690	THC2755690	Unknown	2,916
SOCS2	NM_003877	ref|Homo sapiens suppressor of cytokine signaling 2 (SOCS2), mRNA [NM_003877]	2,908
FAM100B	NM_182565	ref|Homo sapiens family with sequence similarity 100, member B (FAM100B), mRNA [NM_182565]	2,904
ANKRD42	NM_182603	ref|Homo sapiens ankyrin repeat domain 42 (ANKRD42), mRNA [NM_182603]	2,902
GADD45B	NM_015675	ref|Homo sapiens growth arrest and DNA-damage-inducible, beta (GADD45B), mRNA [NM_015675]	2,899
LOC100130910	XM_001715202	gb|PREDICTED: Homo sapiens hypothetical protein LOC100130910 (LOC100130910), mRNA [XM_001715202]	2,894
ACLY	U18197	gb|Human ATP:citrate lyase mRNA, complete cds. [U18197]	2,891
MANBAL	NM_022077	ref|Homo sapiens mannosidase, beta A, lysosomal-like (MANBAL), transcript variant 1, mRNA [NM_022077]	2,864
SORBS2	AF090937	gb|Homo sapiens clone HQ0618 PRO0618 mRNA, complete cds. [AF090937]	2,862
CASP4	NM_033306	ref|Homo sapiens caspase 4, apoptosis-related cysteine peptidase (CASP4), transcript variant gamma, mRNA [NM_033306]	2,852
HCG18	NR_024052	ref|Homo sapiens HLA complex group 18 (HCG18), non-coding RNA [NR_024052]	2,850
ABCB1	NM_000927	ref|Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 1 (ABCB1), mRNA [NM_000927]	2,834
KIAA1875	NR_024207	ref|Homo sapiens KIAA1875 (KIAA1875), non-coding RNA [NR_024207]	2,829
TXK	NM_003328	ref|Homo sapiens TXK tyrosine kinase (TXK), mRNA [NM_003328]	2,818
ENST00000379619	ENST00000379619	ens|Fos-related antigen 2 [Source:UniProtKB/Swiss-Prot;Acc:P15408] [ENST00000379619]	2,809
GOLGB1	X75304	gb|H.sapiens giantin mRNA. [X75304]	2,808
TMCO3	NM_017905	ref|Homo sapiens transmembrane and coiled-coil domains 3 (TMCO3), mRNA [NM_017905]	2,802
THC2682560	THC2682560	Unknown	2,801
PGD	NM_002631	ref|Homo sapiens phosphogluconate dehydrogenase (PGD), mRNA [NM_002631]	2,800
THC2739352	THC2739352	thc|Q3GZE6_9ACTO (Q3GZE6) AAA ATPase, central region, partial (3%) [THC2739352]	2,793
HACL1	NM_012260	ref|Homo sapiens 2-hydroxyacyl-CoA lyase 1 (HACL1), mRNA [NM_012260]	2,782
C5orf26	NR_015370	ref|Homo sapiens chromosome 5 open reading frame 26 (C5orf26), non-coding RNA [NR_015370]	2,777
MC5R	NM_005913	ref|Homo sapiens melanocortin 5 receptor (MC5R), mRNA [NM_005913]	2,776
ERICH1	NM_207332	ref|Homo sapiens glutamate-rich 1 (ERICH1), mRNA [NM_207332]	2,759
PRDM10	NM_199437	ref|Homo sapiens PR domain containing 10 (PRDM10), transcript variant 2, mRNA [NM_199437]	2,754
KLC1	NM_182923	ref|Homo sapiens kinesin light chain 1 (KLC1), transcript variant 2, mRNA [NM_182923]	2,749
ABCB1	NM_000927	ref|Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 1 (ABCB1), mRNA [NM_000927]	2,745
LSS	NM_002340	ref|Homo sapiens lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase) (LSS), transcript variant 1, mRNA [NM_002340]	2,729
RP4-691N24.1	NM_025176	ref|Homo sapiens ninein-like (NLP), mRNA [NM_025176]	2,718
ABCB1	NM_000927	ref|Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 1 (ABCB1), mRNA [NM_000927]	2,706
JUND	X56681	gb|Human junD mRNA. [X56681]	2,693
SKAP1	NM_003726	ref|Homo sapiens src kinase associated phosphoprotein 1 (SKAP1), transcript variant 1, mRNA [NM_003726]	2,681
AUH	NM_001698	ref|Homo sapiens AU RNA binding protein/enoyl-Coenzyme A hydratase (AUH), nuclear gene encoding mitochondrial protein, m	2,666
ANKRD11	NM_013275	ref|Homo sapiens ankyrin repeat domain 11 (ANKRD11), mRNA [NM_013275]	2,666
CELSR3	NM_001407	ref|Homo sapiens cadherin, EGF LAG seven-pass G-type receptor 3 (flamingo homolog, Drosophila) (CELSR3), mRNA [NM_00	2,656
LRRIQ1	NM_032165	ref|Homo sapiens leucine-rich repeats and IQ motif containing 1 (LRRIQ1), transcript variant 2, mRNA [NM_032165]	2,639
THC2656875	THC2656875	Unknown	2,638
DEPDC6	NM_022783	ref|Homo sapiens DEP domain containing 6 (DEPDC6), mRNA [NM_022783]	2,620
PNPLA3	NM_025225	ref|Homo sapiens patatin-like phospholipase domain containing 3 (PNPLA3), mRNA [NM_025225]	2,581
LOC92270	NM_001017971	ref|Homo sapiens V-type proton ATPase subunit S1-like protein (LOC92270), mRNA [NM_001017971]	2,579
RASSF1	NM_170713	ref|Homo sapiens Ras association (RalGDS/AF-6) domain family member 1 (RASSF1), transcript variant C, mRNA [NM_170713	2,574
LOC153546	AK055939	gb|Homo sapiens cDNA FLJ31377 fis, clone NESOP1000087. [AK055939]	2,574
ANKRD42	NM_182603	ref|Homo sapiens ankyrin repeat domain 42 (ANKRD42), mRNA [NM_182603]	2,559
BX100171	BX100171	gb|BX100171 Soares_testis_NHT Homo sapiens cDNA clone IMAGp998C011795, mRNA sequence [BX100171]	2,529

Pigment Cell Melanoma Res. **23**; 238–251

ORIGINAL ARTICLE

Cell cycle inhibitor p21/WAF1/CIP1 as a cofactor of MITF expression in melanoma cells

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KEYWORDS p21/WAF1/MITF/melanoma/CREB

PUBLICATION DATA Received 13 August 2009, revised and accepted for publication 1 January 2010, published online 7 January 2010

doi: 10.1111/j.1755-148X.2010.00670.x

Summary

p21/WAF1/Cip1 (p21), a cyclin-dependent kinase inhibitor, may act as an antioncogene, but may also behave as a tumor promoting factor by inhibiting apoptosis. p21 is also a transcriptional regulator, exerting this activity independently of cyclin-dependent kinases. Increased p21 protein levels were found in a subset of melanomas. However, the mechanism(s) contributing to the tolerance of high p21 levels in melanoma cells remains unexplained. Here, we show that the p21 protein positively regulates the promoter of microphthalmia-associated transcription factor (MITF), a transcription factor which plays a central role in the expression of melanocyte-specific genes, lineage determination, and survival of melanoma cells. p21 activated the MITF promoter-reporter, occupied the promoter in vivo and cooperated with cAMP response element binding protein (CREB) in promoter activation. In addition, p21 knockdown by shRNA resulted in a decrease of MITF protein and promoter activity, and p21 protein levels correlated with MITF mRNA in most cell lines tested. As the p21 gene is a known transcriptional target of MITF, the reciprocal stimulation of transcription may constitute a positive-feedback loop reinforcing MITF expression in melanoma cells. Our results might help explain the tolerance of increased p21 levels found in some melanomas.

Introduction

p21/WAF1/CIP1 has been originally identified as a downstream effector of the p53-mediated block of cell cycle progression. Its physiological function is to act as a universal inhibitor of cyclin-dependent kinases (Cdks). Besides controlling cell proliferation by inhibiting the activity of Cdk-cyclin complexes, p21/WAF1/CIP1 (referred to as p21 hereafter) has been proposed to play a role in transcription regulation. Although p21 can modulate transcription indirectly by inhibiting the phosphorylation of the Rb protein with subsequent blocking of E2F-dependent promoters, the transcription regulatory role of p21 appears to be in many instances

Significance

Inhibitors of Cdks are important negative regulators of cell proliferation frequently inactivated in human cancers. However, the p21(WAF1, CIP1) protein, an effector of the p53 protein that halt the cell cycle after DNA damage, was found overexpressed in many cancer types where it protects malignant cells against apoptotic stimuli. A fraction of melanomas express high levels of p21 which is localized in the nucleus but it is unknown how it could function as an anti-apoptotic factor in these tumors. Our findings suggest a transcriptional function of p21 in melanoma cells. It can support the expression of MITF, a factor critical for survival of normal and malignant melanocytes and a transcription activator of a number of genes including p21. This positive-feedback stimulation may reinforce prosurvival activity of MITF in a subset of melanomas.

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independent of its cell cycle inihibitory activity. p21 down-regulates transcription exerted by several transactivators, including c-myc (Kitaura et al., 2000; Vigneron et al., 2006), STAT3 (Coqueret and Gascan, 2000), and E2F (Delavaine and La Thangue, 1999). Its role in transcription repression has also been described for promoters of survivin (Lohr et al., 2003), Wnt4 (Devgan et al., 2005), tumor necrosis factor receptor (Wang et al., 2005), and cdc25A (Vigneron et al., 2006). In other circumstances, p21 functions as a coactivator stimulating transcription mediated by estrogene receptor α (Fritah et al., 2005; Redeuilh et al., 2002) or NFkB (Kawata et al., 2003; Perkins et al., 1997; Poole et al., 2004).

p21 is endowed with the activity to protect cells against apoptosis elicited by various stimuli. Its cytostatic role in cell cycle regulation is thus extended to a cytoprotective role, and these two functions are coupled presumably because they insure cell viability during the reparation of damaged DNA in growth arrested cells. As the p21 protein has been shown overexpressed in many tumor types, its anti-apoptotic activity may attenuate the efficacy of therapeutic regimens relying on apoptosis induction. This pro-oncogenic activity of p21 has been demonstrated for prostate and breast cancer, gliomas, cervival and ovarian carcinomas and some other tumors, where its increased expression is frequently associated with unfavorable prognosis and poor survival (Abbas and Dutta, 2009; Weiss, 2003).

In pigment cells, the transcription of p21 is activated by MITF (Carreira et al., 2005), a master regulator of melanocyte differentiation and proliferation, whose overexpression, amplification, and mutations have been found in malignant melanoma (Cronin et al., 2009; Garraway et al., 2005; Levy et al., 2006). Several studies demonstrated a positive correlation between p21 protein expression and progression of primary melanomas, whereas low or undetectable levels were observed in benign naevi (Alonso et al., 2004; Maelandsmo et al., 1996; Poyraz et al., 2004; Sparrow et al., 1998; Trotter et al., 1997). Remarkably, high p21 levels were retained even in metastatic lesions (Sparrow et al., 1998; Trotter et al., 1997) and p21 overexpression, in combination with other parameters, has been proposed to be a predictive melanoma marker associated with shorter survival (Alonso et al., 2004). However, whether p21 plays any cell cycle regulatory role in malignant melanocytes and why its relatively high protein level is tolerated in some melanoma cells remains unresolved.

To examine the potential cell cycle-independent role of p21 in transformed melanocytes, we investigated the possibility that p21 might participate in expression of pigment cell-specific genes. We show herein that p21 transactivates the MITF promoter, occupies this promoter in cells, and p21 knockdown down-regulates MITF promoter activity and protein level. As p21 is a transcriptional target of MITF, a mutual regulation between these two factors may represent a positive-feedback loop which may help to maintain elevated MITF level required for the expression of anti-apoptotic and pro-proliferative genes, thus contributing to the survival of melanoma cells.

Results

p21 stimulates transcription of the MITF promoter-reporter and MITF target promoters

The presence of apparently tolerated p21 protein in malignant melanoma cells and its regulatory role in transcription described in other cell types prompted us to investigate whether p21 has any transcriptional function in melanoma. We first asked whether p21 has any regulatory role in transcription of melanocyte-specific promoters. Therefore, we tested the influence of p21 coexpression on the activity of promoter-reporters for MITF and its three known target genes, tyrosinase, melastatin, and TRP1 in four melanoma cell lines MeWo, SK-MEL-28, SK-MEL-5, and B16-F10. Figure 1A shows that exogenous p21 stimulated the activity of all promoters, with MITF and melastatin being more responsive, both activated up to sixfold by p21 when compared with the empty vector. Generally, the activation was more evident in p21 non-expressing cell lines MeWo and SK-MEL-28 than in p21-positive lines SK-MEL-5 and B16-F10 (for p21 expression, see Figure 2A, B), where the melastation promoter was stimulated only about twofold. In B16-F10 cells, MITF promoter was also effectively activated by p21 (Figure 1A). These results indicate that p21 participates in the transactivation of MITF promoter, and stimulation of target promoters might be a downstream consequence of transiently elevated MITF in transfected cells.

Activation of MITF promoter by p21 is specific

As above, p21 has been shown previously to display both positive and negative regulatory effects on different promoters. To confirm that the action of p21 on MITF and other promoters in our studies was specific, we examined its effect on the survivin promoter, which is known to be p53-repressible through p21 (Lohr et al., 2003). Indeed, cotransfection of the p21 expression plasmid strongly repressed the survivin promoter-reporter in SK-MEL-28 cells (Figure 1B), and the same was seen in SK-MEL-5 cells (results not shown). We further tested whether the GAL-linked p21 protein did not activate transcription non-specifically when tethered to a promoter. As shown, the activity of the 5xGAL-Luc promoter was slightly inhibited by GAL-p21 in a dose-dependent manner in SK-MEL-28 cells (Figure 1B), MeWo and B16-F10 cells (results not shown), confirming that the promoter-tethered p21 is unable to activate transcription non-specifically. We next assayed the effect of p21 on the promoter containing the NFkB-responsive elements, which was shown previously to be induced by p21 in fibrosarcoma cells (Poole et al., 2004). In melanoma cells, however, the

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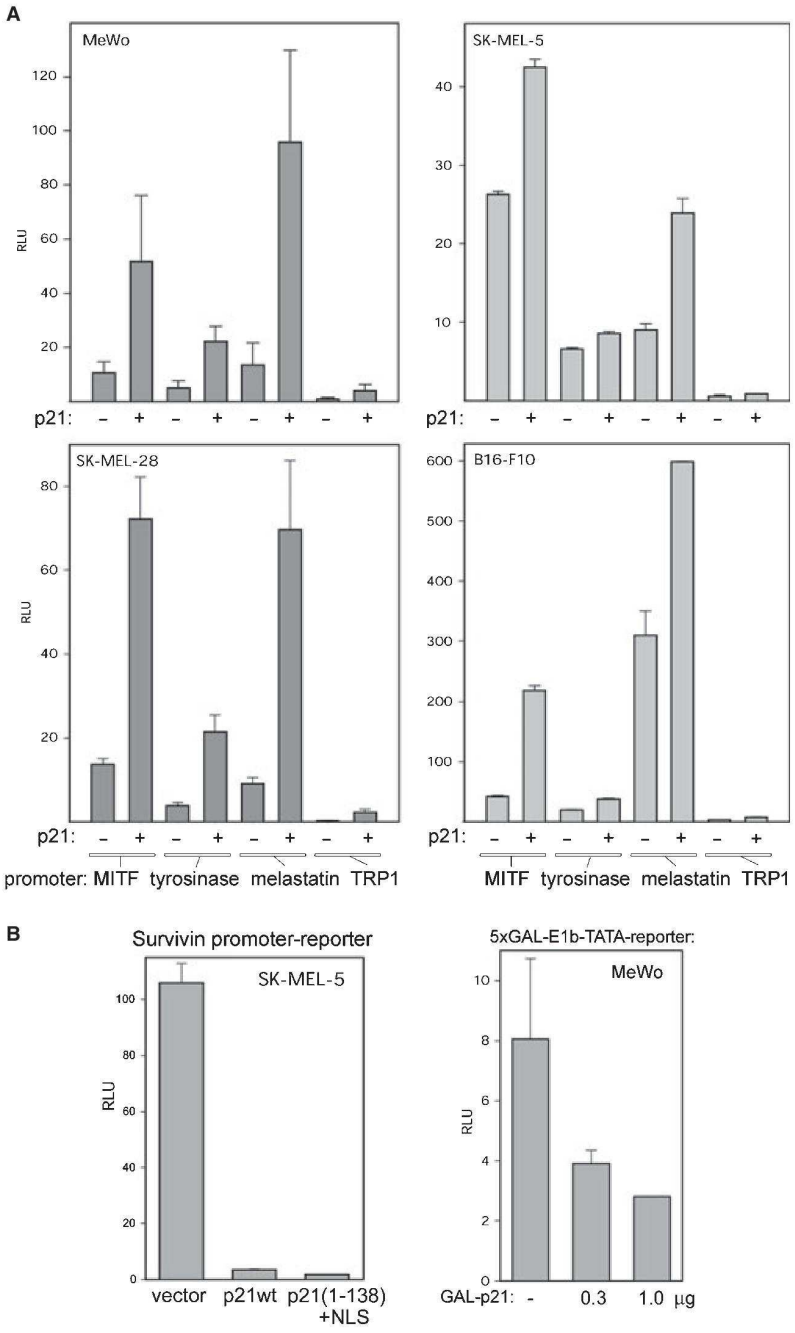

Figure 1. p21 activates melanocyte-specific promoters but does not function as a general transactivator in melanoma cells. (A) Promoter-luciferase constructs (0.4 μg) for MITF, tyrosinase, melastatin, and TRP1 were cotransfected with a control vector or a human p21 expression vector (1 μg) in melanoma cell lines and luciferase activities were determined. (B) Inhibition of non-melanocyte promoters by p21. Left, p21 inhibits the survivin promoter. Cells were transfected with the reporter (0.2 μg) and expression plasmids (1.0 μg) and luciferase activity estimated in cell extracts. Right, GAL-p21 fusion protein represses transcription from the 5xGAL-Luc promoter. Cells were transfected with the reporter plasmid (0.2 μg), GAL-p300 fusion plasmid (0.2 μg) and an empty vector (–) or an increasing dose of p21 expression plasmid. Both GAL4 and GAL4-p21 were expressed in cells (not shown). Each experiment was carried out at least three times and one experiment is presented. Bars indicate +SE of duplicate samples. RLU, relative luciferase units.

NFkB transcription was inhibited about threefold (data not shown). Together, these data show variable and specific effects on different promoters and rule out the possibility that p21 could activate MITF promoter non-specifically in melanoma cells.

Expression of p21 protein in melanoma cells and normal melanocytes

We next analyzed human melanoma cell lines for p21 expression and found that about half of them expressed moderate or high p21 levels, whereas some cell lines lost its expression (Figure 2A). This variability of p21 protein expression in melanoma cell lines was observed also in previous studies (Maelandsmo et al., 1996; Vidal et al., 1995). Contrary to the p21 protein, Cdk2 and its cyclin partner, cyclin E, were uniformly expressed in all cell lines (Figure 2A), consistent with the previously described importance of Cdk2 for proliferation of melanoma cells (Du et al., 2004). We also determined whether p21 is expressed in normal human epidermal

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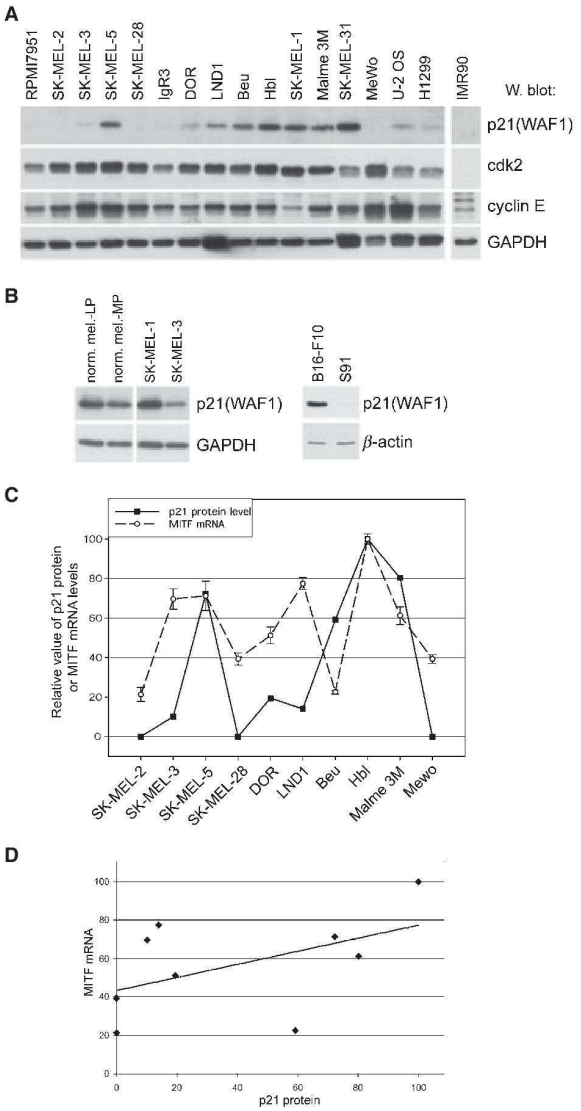

Figure 2. Western blot analysis of p21 protein expression in melanoma cell lines and normal human melanocytes. (A) Expression of p21, Cdk2 and cyclin E in melanoma cells. Protein levels in whole-cell extracts were analyzed by immunoblotting with antibodies against p21, cdk2, cyclin E, and GAPDH. Two melanoma cell lines (RPMI7951 and IgR3) are dedifferentiated and do not express MITF and downstream markers. For comparison, the non-melanoma tumor cells U2OS (osteosarcoma), H1299 (lung carcinoma), and diploid human fibroblasts (IMR90) are shown. (B) Left, normal melanocytes (lightly pigmented, LP, and moderately pigmented, MP) were analyzed for p21 and GAPDH protein levels. To compare the signal between normal melanocytes and melanoma cells, SK-MEL-1 and SK-MEL-3 cell extracts were immunoblotted together with melanocytes. Right, two mouse melanoma cell lines, B16-F10 and Cloudman S91, were analyzed for p21 protein expression. (C) Correlation of MITF mRNA and p21 protein levels in human melanoma cell lines. RNA and protein were isolated and analyzed as described in Materials and Methods. Expression levels of MITF in cell lines were estimated by real-time PCR method and standard errors from triplicate samples are shown. Analysis was performed in two separate experiments from which similar results were obtained, and one experiment is presented. Expression values for MITF were normalized to β-actin. p21 protein values were obtained by software analysis of Western blot signals shown in Figure 2A and normalized to values of GAPDH. The highest level of both parameters was set as 100. (D) Linear regression depicting a weak correlation (R = 0.52) between p21 protein abundance and MITF RNA in ten melanoma cell lines shown in (C).

melanocytes in culture. As shown in Figure 2B, normal human melanocytes also express p21 protein, and its level is high even in lightly pigmented melanocytes, which grow faster than moderately pigmented melanocytes. Further, mouse melanoma cells B16-F10, despite the fact that they proliferate rapidly, express abundant p21 protein when compared with another mouse melanoma cell line Cloudman S91 (Figure 2B) which has slightly lower proliferation rate. Thus, the expression pattern of p21 in tumor cells and normal melanocytes suggest that abundant p21 may not restrict proliferation in pigment cells. Because p21 is a p53 target gene, we also estimated the protein levels of p53 in melanoma cell lines. Clearly, p21 protein expression was independent of p53 abundance as all melanoma cell lines, with the only exception of the RPMI7951 line which was p53-null, revealed moderate or high p53 protein (Figure S1).

p21 protein and MITF mRNA levels in melanoma cell lines

To further substantiate the results with promoter-reporters suggesting that p21 participates in MITF expression, it was relevant to evaluate whether the p21 protein abundance correlates with MITF mRNA. In this experiment, we excluded two MITF-negative, undifferentiated cell lines, RPMI7951 and IgR3, which do not express MITF and other melanocyte markers (Vachtenheim et al., 2001), an atypical line SK-MEL-1 growing in suspension, and an extremely slowly proliferating SK-MEL-31 cells with a very high p21 protein level. Thus, of the cell lines shown in Figure 2A, only adherent, normally proliferating differentiated cell lines, i.e. those displaying typical features of melanoma cells in culture, were chosen for analysis. Real-time PCR was used to estimate the mRNA levels of the melanocyte-specific MITF-M isoform and the signal of the p21 protein on Western blot (Figure 2A) was quantified by a densitometric analysis. Interestingly, a similar trend between both parameters was found in seven out of ten cell lines (Figure 2C), whereas SK-MEL-3 and LND1 cells had low p21 protein and a relatively high MITF mRNA, and Beu cells revealed low MITF mRNA and abundant p21 protein (linear regression between both parameters is shown in Figure 2D). Noteworthy, the MITF mRNA fluctuation among cell lines was much lower (within the fivefold range) than the differences in p21 protein levels, and was not directly reflected by the MITF

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protein (Figure S2). These data suggests that mainly post-transcriptional and/or post-translational mechanisms are important in regulation of MITF protein level and confirms the relevance of MITF mRNA determination in this experiment. Further, equal levels of MITF protein were found in human melanocytes LP and MP and a more abundant MITF was observed in mouse S91 cells than in B16-F10 (not shown), demonstrating no direct correlation between MITF and p21 in these cells. To complement these studies, we also determined mRNA levels of the prototypal MITF target, tyrosinase, and compared them with MITF mRNA values. As shown in Figure S3, although these two parameters revealed a similar trend in many cell lines, the weak correlation was even less significant (regression analysis not shown) than that obtained with p21 protein and MITF mRNA. In aggregate, the data suggest that MITF mRNA reflects the p21 protein levels in human melanoma cell lines and corroborate the reporter assays together implying MITF as a gene potentially co-regulated by p21 protein.

p21 activates MITF promoter independently of its ability to derepress the p300/CBP CRD1 domain

The amino-terminal portion of p21 is sufficient to induce cell cycle arrest and contains one of the two cyclin-binding sites and a Cdk-binding site (schematically shown in Figure 3A). The carboxy-terminal cyclin-binding site is dispensable for inhibiting the Cdk activity and overlaps with a region that associates with PCNA to inhibit DNA replication (Dotto, 2000). To determine which region of p21 was required for MITF promoter activation, cotransfections were performed in B16-F10 mouse melanoma cells using different p21 deletion mutants. Transfection of wild-type p21 and its two mutants, one with a deletion of the Cdk2 binding domain (aa 52–57) and the other expressing only the first 133 amino acids, activated transcription more than ninefold. By contrast, deletion of the amino-terminal cyclin-binding site (amino acids 17–23) abolished the transactivation effect (Figure 3B). Western blot analysis confirmed the expression of p21 proteins; the Δ17–23 protein expression was lower and the Δ52–57 mutant showed weak expression, but despite this, the Δ52–57 mutant still activated the promoter (Figure 3C). Thus, the mutational analysis revealed that the C-terminus is dispensable, while the N-terminus with the cyclin-binding motif is required for promoter stimulation.

MITF expression in melanocytes is regulated by several transcription factors, including cAMP response element binding protein (CREB) and LEF-1, both of which are known to associate with CBP and p300 coactivators to enhance transcription of target genes (Johannessen et al., 2004; Miyagishi et al., 2000). Thus, although not reported as yet, the melanocyte-specific MITF isoform might require p300/CBP for expression. Transcriptional coactivators p300 and CBP have been reported to harbor an intrinsic repression domain that counteracts their transactivation function (Gregory et al., 2002; Snowden et al., 2000). This short domain, named CRD1 (residues 1017–1029 in p300), contains sites which can be modified by binding of SUMO proteins. Subsequent recruitment of histone deacetylase 6 (HDAC6) to SUMO-modified CRD1 mediates transcriptional repression (Girdwood et al., 2003). Importantly, the CRD1 is sensitive to overexpression of p21, which thus counterbalances the repressive effect of CRD1 resulting in a strong enhancement of the p300-mediated coactivation (Garcia-Wilson and Perkins, 2005; Girdwood et al., 2003; Gregory et al., 2002; Snowden et al., 2000).

Although the molecular mechanism of the p21-mediated CRD1 derepression is unknown, it requires the C-terminal region of p21 and is independent of p21 binding to cyclins or Cdks (Garcia-Wilson and Perkins, 2005). As the carboxy-terminal region of p21 was dispensable for MITF promoter activation (Figure 3B), we wanted to verify that the stimulatory action did not involve CRD1 derepression. We therefore analyzed which domains were necessary for the derepression of CRD1 in B16-F10 cells. The 5xGAL-Luc reporter plasmid and a vector expressing GAL-p300(1-1303) were contransfected with the wt p21 and its mutants and the reporter activity was estimated. In this case, the C-terminus, but not the cyclin binding box was necessary for activation, indicating that MITF promoter indeed utilizes distinct regions of p21 than are necessary for the CRD1 derepression, in the same cells (Figure 3D). The expression level of p21 proteins was similar as shown in Figure 3C, indicating that a lower expression efficiency of the Δ52–57 mutant was again sufficient for transactivation.

The HDAC6, but not other HDACs, has been previously implicated in repression by the CRD1 domain (Girdwood et al., 2003). To further confirm that a derepression of CRD1 was not involved in p21-mediated activation, the MITF promoter, or the GAL-Luc promoter with the GAL-p300(1-1303) plasmid, were cotransfected together with the HDAC6 expression vector and p21 constructs. As expected, HDAC6 counteracted the p21-mediated derepression, whereas it slightly activated MITF promoter in the presence or absence of wt p21 (Figure 3E). The activation elicited by p21(1-133)NLS was only mildly compromised by cotransfection of HDAC6. Taken together, the results convincingly show that the activation of MITF promoter by p21 does not proceed via the derepression of the p300 CRD1 domain.

MITF promoter is not stimulated through a general inhibition of Cdks

Because the p21 mutant Δ17–23, which is impaired for association with cyclin E and cell cycle inhibiting activity (Delavaine and La Thangue, 1999; Wohlschlegel et al., 2001) failed to activate MITF promoter (Figure 3B), we assessed whether the transcriptional stimulation was linked to inhibition of cyclin-dependent kinases.

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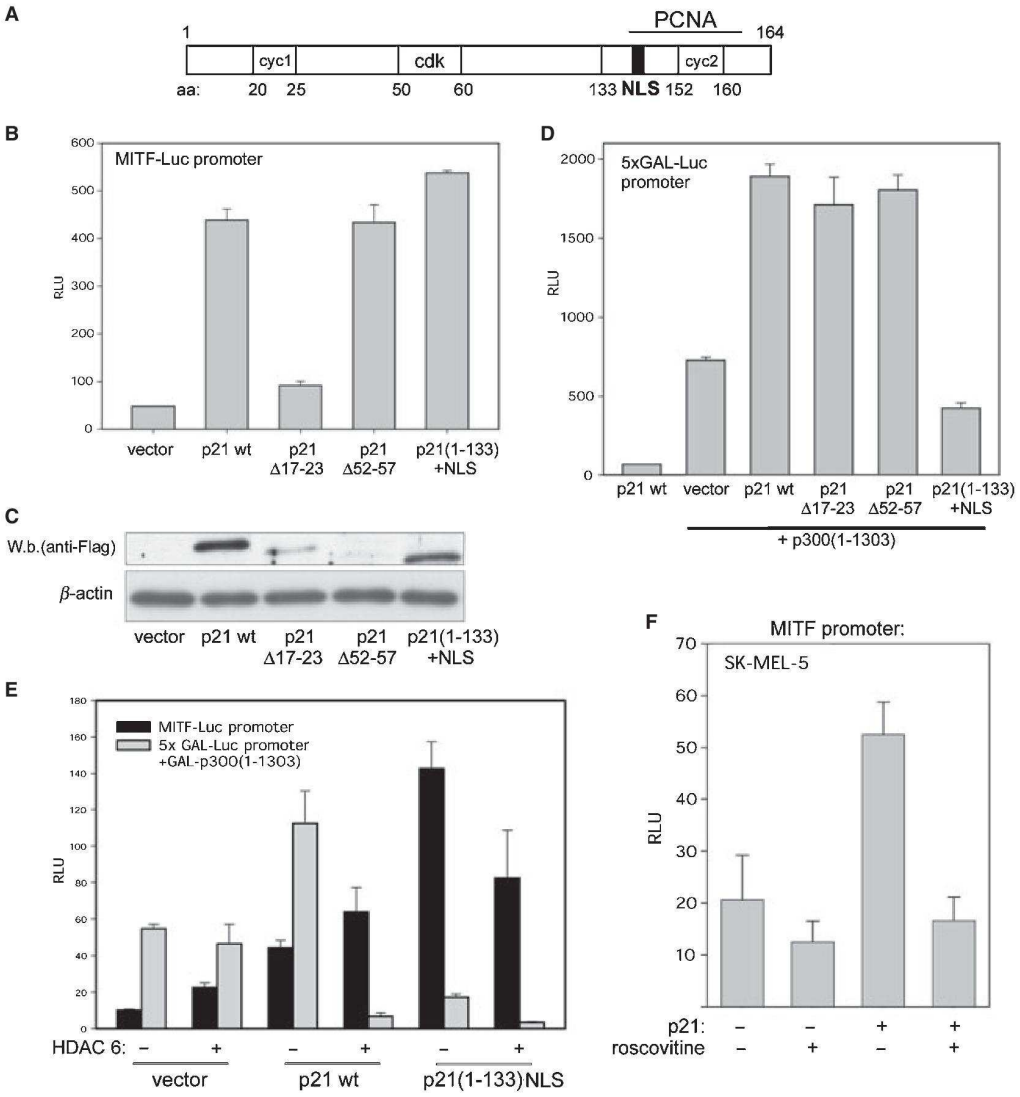

Figure 3. Derepression of the p300 CRD1 domain by p21 is not necessary for the activating effect of p21 on MITF promoter and inhibition of cdks by roscovitine does not activate the promoter. (A) Diagram of regions in the human p21 molecule (164 aa) showing two cyclin-binding sites (cyc1 and cyc2), the cdk-binding site (cdk), the PCNA binding region, and nuclear localization sequence (NLS). The domains are conserved in the mouse protein. (B) Activation of MITF promoter by p21 protein mutants. B16-F10 melanoma cells were cotransfected with the human MITF promoter plasmid (0.4 μg) and the indicated FLAG-tagged mouse p21 expression vectors (1 μg). (C) Expression of FLAG-p21 proteins in experiment shown in panel (B) was determined by Western blot analysis with the anti-FLAG antibody. (D) Stimulation of the GAL-p300(1-1303) activity by coexpression of p21 protein mutants. Cells were cotransfected with the human MITF promoter and the indicated FLAG-tagged p21 expression vectors. The expression level of p21 proteins was similar as shown in panel (C). (E) HDAC6 counteracts derepression of CRD1 but does not inhibit stimulation of MITF promoter by p21. B16-F10 melanoma cells were cotransfected with MITF promoter (0.4 μg) or 5xGAL-Luc promoter (0.2 μg) and p21 constructs as indicated, and luciferase activity was determined. (F) Roscovitine decreases the activity of MITF promoter. Cells were transfected with the MITF-luc promoter plasmid (0.4 μg) and 1.0 μg of FLAG-p21 vector and treated with vehicle (–) or roscovitine (15 μM) for 16 h before harvesting. In panels (B), (D), (E), and (F), luciferase activity was determined in three independent experiments from which one is presented. RLU, relative luciferase units. Bars are shown with a standard error of duplicate samples.

However, the MITF promoter activity was slightly repressed, rather than activated, by the addition of a Cdk inhibitor roscovitine in vector-transfected cells (Figure 3F). Roscovitin suppressed the activity of the p21-stimulated promoter even more prominently, about threefold. These data indicate that a complete inhibition of Cdk2 (and Cdk1) activity is not responsible for promoter activation. More likely, p21 may activate MITF promoter in a specific manner rather than through a global inhibition of cdks.

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p21 cooperates with the cAMP-CREB pathway in upregulation of MITF promoter

The MITF gene is cAMP-responsive and its proximal promoter harbors a CRE consensus. Because p21 has been suggested previously as a co-stimulator of cAMP signaling in non-melanocyte cell types (Drdova and Vachtenheim, 2005; Kawata et al., 2003), we asked whether p21 and CREB could cooperate in MITF promoter activation in B16-F10 melanoma cells. As shown in Figure 4A, both CREB and DN-CREB (the S133A mutant) slightly activated the promoter in the absence of p21. In contrast, upregulation by p21 was further potentiated by CREB, about fourfold, whereas the DN-CREB plasmid elevated the activity only marginally in this case. Thus, cotransfected p21 and CREB induced MITF promoter in a cooperative fashion. In a control experiment, CREB activated 5xCRE-luc promoter about twofold, while p21 had only a minimal effect (Figure 4A, insert). We also noticed that the CRE-mutated promoter-reporter retained only a residual activity in several cell lines tested (Drdova and Vachtenheim, 2004; data not shown). To determine whether the p21-mediated activation depends on CREB consensus motif, we cotransfected the p21 plasmids and the wt or a CRE-mutated MITF promoter and determined luciferase activity. Whereas both p21 and p21(1-133)NLS stimulated the wt promoter about sixfold, the low activity of the mutated promoter was augmented up to about 2.5-fold (Figure 4B). The Δ17–23 mutant activated wt and mutated promoters negligibly. p21 proteins were expressed (Figure 4C), albeit the Δ17–23 mutant less strongly (not shown, see also Figure 3C). Mutation of other binding sites in the promoter (Sox10, Pax3) did not substantially decrease the promoter activity and these promoters behaved similarly as wt upon p21 cotransfection (data not shown). Taken together, a substantial part of activation brought about by p21 relied on CREB-mediated transactivation.

The MITF downstream gene tyrosinase is responsive to cAMP-elevating agents through the MITF-binding sites in its promoter (Bertolotto et al., 1998). Thus, we investigated whether p21 overexpression can increase either MITF or tyrosinase promoter activity induced by forskolin. The p21 was cotransfected with the respective promoter-reporter and cells were treated with forskolin 12 hrs before harvesting. In this setting, p21 only slightly increased the forskolin-stimulated MITF promoter activity and the p21-activated promoter could not be further upregulated by forskolin. However, the tyrosinase promoter was further potentiated (more than twofold) by p21 in the presence of forskolin (Figure 4D). Because the effect on tyrosinase promoter is mediated by MITF, these results corroborate the above findings and suggest that overexpressed p21 cooperates with the cAMP-CREB pathway in augmenting MITF expression. To elucidate whether endogenous CREB participates with ectopic p21 in pigment cell-specific promoter activation, a vector expressing functional shRNA directed to CREB (Dobroff et al., 2009) was cotransfected with the p21 plasmid and either MITF or melastatin promoter. Whereas the activity of both promoters was unchanged or slightly increased after transfection of control shRNA vector, sh-CREB diminished the activity of both promoters (Figure 4E). These data further confirm the involvement of CREB signaling in p21-mediated activation of MITF promoter.

MITF promoter is occupied by p21 in cells

Next, we sought to determine whether the p21 protein is recruited to MITF promoter in melanoma cells. For this purpose, we used Hbl cell line as these cells express abundant p21 protein, and the promoter-associated p21 was determined by chromatin immunoprecipitation. Amplification of the MITF promoter was performed with primers spanning the region containing the binding sites for several transcription factors known to be MITF activators (Figure 5A). DNA pulled down by the anti-p21 antibody showed specific association of p21 with the promoter region, while non-immune IgG failed to precipitate any DNA (Figure 5B, upper panel). Significantly, acetylated histone H4 also occupied the promoter, and the amount of DNA immunoprecipitated by anti-p21 was comparable to that pulled down by anti-histone H4 antibody (Figure 5B, upper panel). In control experiments, only acetylated histone H4 antibody immunoprecipitated the unrelated GAPDH promoter (Figure 5B, middle panel) and no positive signal was seen with the anti-p21 antibody in 501mel cells expresing low level of p21 (Figure 5B, lower panel). These results suggest that p21 is associated with the initiation complex at the MITF promoter.

Effect of acute knockdown of MITF or p21 on reciprocal protein level in melanoma cells

To assess the effect of p21 protein downregulation on MITF promoter activity, we knocked-down the expression of p21 in SK-MEL-5 cells using plasmids encoding three different targeting shRNA sequences or a non-targeting sequence as a control. All three p21-directed constructs decreased the MITF promoter activity in the range of 30–40 percent (Figure 6A, lanes 2–4). Because SK-MEL-5 cells are inefficiently transfected, parallel dishes were transfected also with puromycin resistance plasmid and selected for additional 5 days in puromycin to evaluate the knockdown efficiency. The p21 expression was down-regulated approximately two- to three-fold (Figure 6A, lower); however, the endogenous MITF protein remained unchanged in this experiment (not shown). These data indicate that the endogenous p21 protein participates in activation of the transfected promoter.

Because the p21 gene was shown to be a transcriptional target of MITF (Carreira et al., 2005), we investigated a possible mutual regulation of expression

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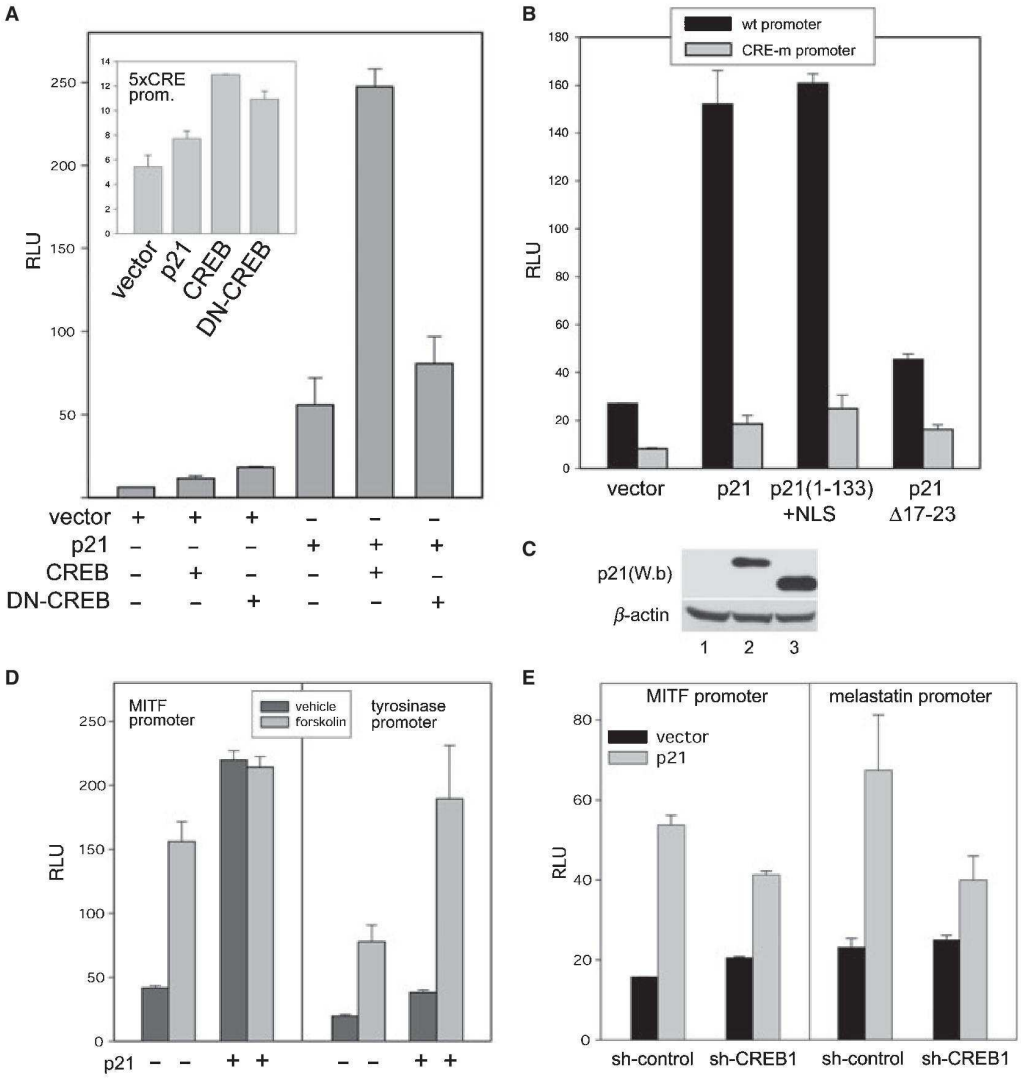

Figure 4. p21 and CREB signaling act synergistically to augment MITF promoter activity. (A) p21 and CREB cooperatively activate MITF promoter. MITF-luc construct (0.4 μg) was transiently cotransfected with 0.6 μg of an empty FLAG vector (left 3 bars) or the FLAG-p21 plasmid (right 3 bars), together with a vector or either CREB or DN-CREB expressing plasmids (0.6 μg) into B16-F10 cells. Insert: CREB but not p21 activates classical 5xCRE-luc promoter. The same amounts of expression plasmids as in (A) were transfected. (B) Mutations in CRE motif reduced the promoter activity and abrogated most of its induction by full-length p21 and carboxy-end-truncated construct p21(1-133)NLS. The Δ17–23 p21 mutant activated both promoters <twofold. A total of 1 μg of indicated p21 plasmids were transfected together with 0.4 μg of wt or mutated promoter, as indicated. (C) Wt FLAG-p21 (lane 2) and FLAG-p21(1-133) (lane 3) used in experiments shown in (B) are expressed in B16-F10 cells. Empty vector was transfected in lane 1. The same expression of FLAG-p21 proteins was seen in experiments in (A) and (D). Western blot was detected with antibodies against FLAG or β-actin. (D) B16-10 cells were transfected with promoter-reporter plasmid (0.4 μg) and an empty vector or p21 construct (1 μg) and treated with DMSO or 20 μM forskolin for 12 h before analysis. (E) Sh-CREB but not the sh-control plasmid decreased the p21-stimulated activity of MITF or melastatin promoter. SK-MEL-5 cells were used as a higher effect of CREB knockdown was seen in these cells when compared with B16-F10 cells despite the shared target sequence in mouse and human RNA (not shown). All experiments were performed in duplicates and each experiment was repeated at least two times. RLU, relative luciferase units. Error bars indicate +SE.

between MITF and p21. Further, to assess a more acute effect of shRNA-mediated p21 knockdown and to efficiently transduce all cells, we infected chosen p21-positive melanoma cells, Hbl and SK-MEL-5, with lentiviruses encoding shRNA directed to either MITF or p21, and analyzed proteins 2 days later. More than 90% of Hbl cells and nearly 100% of SK-MEL-5 cells were transduced as estimated by GFP positivity (data not shown). In Hbl cells infected with the p21-directed lentivirus, we could not observe any change in MITF protein, despite a marked decrease of p21 (Figure 6B). Reciprocally, donwregulation of MITF was more effective and

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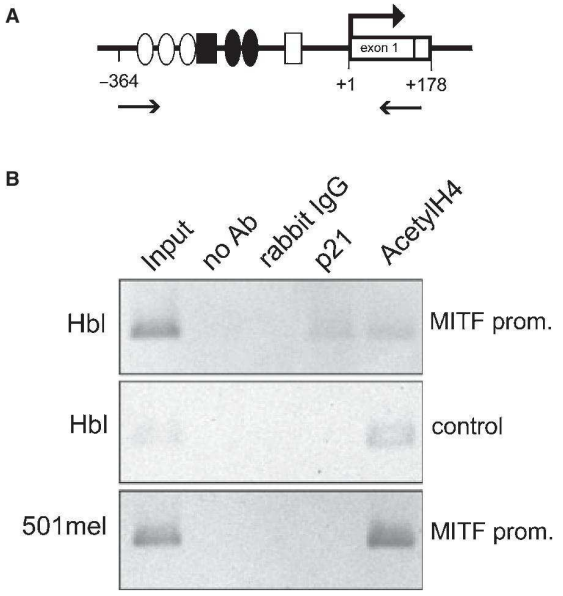

Figure 5. p21 protein physically occupies MITF promoter. (A) Schematic view of the melanocyte-specific MITF promoter showing the transcription start, a melanocyte-specific first exon, and binding sites within the promoter: open ovals, Sox10 sites; filled ovals, LEF/TCF sites; filled rectangle, Pax3 site; open rectangle, CRE site. Arrows mark the position of primers used to amplify the promoter region in chromatin immunoprecipitation assay. (B) Upper, ethidium bromide-stained agarose gels of PCR reaction products detecting the MITF promoter region from the p21 antibody and control IgG immunoprecipitated DNA in Hbl cells. Middle, control region of the GAPDH gene amplified by PCR reaction from samples as for upper panel. Three experiments were performed with similar results. Lower, MITF promoter region from the p21 antibody and control IgG recovered DNA from 501mel cells, which express low amount of p21 (not shown).

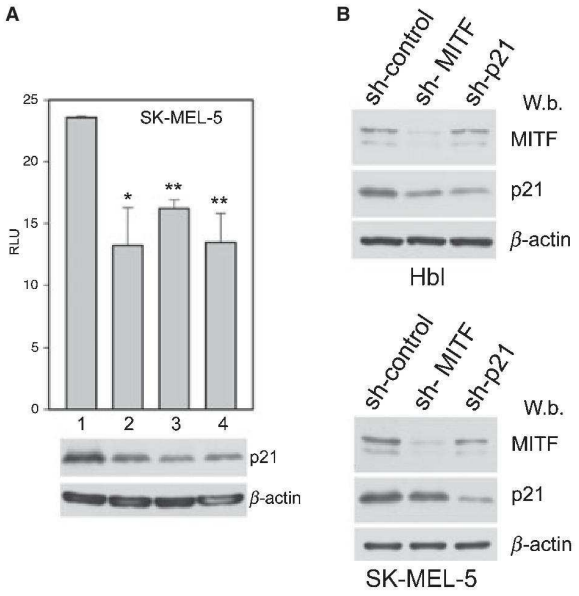

Figure 6. Effect of shRNA-mediated downregulation of p21 on MITF promoter activity and reciprocal regulation of p21 and MITF at endogenous levels. (A) MITF-Luc construct (0.4 μg) and each of the shRNA constructs (1.0 μg) were transiently cotransfected in SK-MEL-5 melanoma cells and luciferase activity was determined 2 days later. Bar 1, non-targeting construct in pSUPER.puro vector; bar 2, p21-specific shRNA construct in pSUPER.puro; bars 3 and 4, shRNA plasmids encoding shRNA, purchased from Origene (constructs 1 and 4). Asterisks indicate P values (*P = 0.078; **P < 0.05) for comparison between control (bar 1) and experimental samples (bars 2–4). Lower panel, Western blot of protein extracts from SK-MEL-5 cells transfected as above and selected 5 days in 1 μg/ml puromycin to determine the p21 depletion. The level of p21 knockdown was slightly underestimated because antibiotic selection did not remove all non-transfected cells. (B) Mutual regulation of p21 and MITF in two p21-expressing melanoma cell lines. Hbl (upper panel) or SK-MEL-5 (lower panel) cells were infected with lentiviruses (5 MOI) as described in Materials and methods and protein extracts were immunoblotted with antibodies against MITF, p21, or β-actin.

led to a slight decrease of the p21 protein (Figure 6B). In SK-MEL-5 cells, the MITF knockdown was also effective but produced only a minute decrease of its target p21. Contrary to Hbl, p21 knockdown in SK-MEL-5 cells resulted in an evident reduction of the MITF protein (Figure 6B). Both MITF and tyrosinase mRNA estimated by real-time polymerase chain reaction (PCR) were also slightly, but significantly ($P < 0.05$) reduced in the sh-p21-transduced SK-MEL-5 cells (by 14 and 10 percent, respectively, data not shown). Thus, these data suggest that expression of MITF and p21 may be reciprocally regulated in p21-positive melanoma cells.

Discussion

p21 is a multifunctional protein with several independent activities including cell cycle arrest and has been implicated, together with p16INK4A and p14ARF, in the senescence of melanocytes. In this report, we have demonstrated that p21 has a regulatory role in transcription activation of the melanocyte-specific MITF promoter in melanoma cells. This role is accomplished via the N-terminal part of p21, where the cyclin-binding site is necessary for promoter activation (Figure 3). Numerous transcription factors utilize coactivators p300 and CBP, which function as universal regulators of gene expression. p21 protein is capable of stimulating the p300/CBP-mediated transactivation by counteracting the function of the CRD1 repression domain. We have suprisingly found that this mechanism is not involved in MITF promoter activation by p21 (Figure 3). This finding does not rule out the involvement of p300/CBP in promoter stimulation by p21, since a modulation of p300/CBP activity by p21 can still be accomplished without influencing CRD1-mediated repression. Such an enhancement of the p300-coactivated transcription was documented previously, and this effect of p21 was mediated by inhibiting Cdk2 kinase activity in the

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p300-associated Cdk2-cyclin E complex (Perkins et al., 1997). Similarly, another report has shown that p21 can inhibit phosphorylation of CBP, thus increasing its activity and hence transcription of estrogen receptor-α target genes (Redeuilh et al., 2002).

Several transcription factors positively regulate MITF promoter and the relative constribution of each of them may differ among melanoma cell lines. The p21 does not bind DNA and can influence transcription only as a coregulator present in complexes at transcription initiation sites. We demonstrated here that the endogenous p21 indeed occupied MITF promoter in vivo (Figure 5). We observed an oposite effect of two Cdk inhibitors, p21 and roscovitin, on the activity of MITF promoter-reporter (Figure 3F). Although this seems paradoxical, it may be explained by a presumed specific regulatory role of p21 in the initiation complex on the promoter. The overall inhibition of Cdk activity after overexpression of p21 in the cell presumably does not play a role in activation, as can be inferred from the fact that roscovitine does not stimulate but slightly inhibits the promoter. Hypothetically, since the two p21 mutants (Δ17–23 and Δ52–57), which are both deficient for inhibiting the cdk activity (Delavaine and La Thangue, 1999), behave differently when tested for MITF promoter activation (Figure 3B), the stimulatory role of p21 eventually may not depend on Cdk inhibition. Rather, as only the cyclin non-binding mutant Δ17–23 lost the activity, the coactivator function of p21 might require a specific recruitment of cyclins to the protein complex at the promoter, which is a possibility that remains to be investigated.

Intriguingly, the effect of p21 on endogenous MITF was demonstrated by the finding that downregulation of endogenous p21 or MITF proteins reciprocally decreased the level of each other, albeit this effect was not ambilateral in both cell lines tested (Figure 6). This might also be due to a more efficient downregulation of MITF expression in comparison with p21. The MITF protein decreased in SK-MEL-5 cells but not in Hbl cells treated with lentivirus expressing p21-directed shRNA, while the effect of MITF-directed shRNA on p21 was seen in both cell lines, more prominent being in Hbl cells (Figure 6). The suggested mutual regulation of p21 and MITF was further supported by demonstrating that the levels of p21 protein and MITF mRNA correlated in seven out of ten melanoma cell lines (Figure 2C).

Previously, studies have demonstrated that p300/CBP proteins function as cofactors of MITF-regulated downstream genes (Sato et al., 1997). The activation of several MITF target promoters by p21 is suggestive of their regulation by induced endogenous MITF (Figure 1A) and implies an involvement of p300/CBP. Further, we have shown that the key stimulator of melanogenesis, the cAMP-CREB pathway, cooperates with p21 to stimulate MITF and its downstream promoters for tyrosinase and melastatin (Figure 4). Although the effect of cAMP on tyrosinase expression is mediated through MITF, and we observed a synergistic effect of p21 and forskolin on the tyrosinase promoter, we cannot entirely rule out that p21 coactivates the downstream MITF promoters also directly, perhaps by augmenting the p300/CBP function as well.

Previous immunohistochemical analyzes as well as the results described here imply that the presence of p21 apparently does not interfere with cell cycle progression in p21-positive melanoma cells. We have observed that the p21 protein is expressed in low pigmented, more rapidly proliferating normal human melanocytes (Figure 2B). In addition, we determined the growth rates of two clones of SK-MEL-5 cells in which p21 was down-regulated by stable expression of shRNA. Although MITF level in these clones remained unchanged (data not shown), we observed decelerated proliferation when compared with the control shRNA-expressing cells (Figure S4). Intriguingly, previous study has shown that mouse p21−/− melanocytes are susceptible to cell death after the withdrawal of phorbol ester from the culture medium and p21 is induced when quiescent melanocytes are growth-stimulated with this agent (Halaban et al., 1998). Furthermore, p21 prevents apoptosis induced by overexpression of p53 in melanoma cells (Gorospe et al., 1997). All these data are in accord and suggest that p21 has a function which is different from cell cycle inhibition in the melanocyte system, pointing to its likely role in cell survival.

p21 expression was seen throughout melanoma progression, by immunohistochemistry, and the most striking difference was the upregulation of p21 protein in primary melanoma lesions when compared to nevi. Importantly as well, although a higher expression of p21 in thinner primary melanomas has also been described (Karjalainen et al., 1999), most studies show a significant correlation between p21 expression and tumor thickness in superficial spreading melanomas (Alonso et al., 2004; Maelandsmo et al., 1996; Poyraz et al., 2004; delaSerna et al., 2009; Trotter et al., 1997). This stage of tumor represents a critical point in melanoma progression when tumor cells are acquiring the keratinocyte-independent growth, and an increased level of MITF might be required to resist apoptosis during subsequent invasion into the collagen-rich dermis.

In general, the mechanisms by which p21 inhibits apoptosis involve the inhibition of Cdk activities, transcriptional repression of pro-apoptotic genes (or coactivation of transcription of anti-apoptotic genes), and inhibition of caspases after a mislocalization to the cytoplasm (Asada et al., 1999; Janicke et al., 2007; Suzuki et al., 1998; Weiss, 2003). Previous immunohistochemical analyzes of p21 protein showed nuclear staining in melanoma cells. We have also observed that p21 was localized in the nucleus in melanoma lines (data not shown), indicating that the prosurvival activity of p21 in melanoma is associated with its function in the cell nucleus. Here, we suggested that p21 may have an

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anti-apoptotic role by augmenting MITF expression in melanoma cells. Another potential mechanism responsible for the anti-apoptotic effect of p21 in melanoma cells may involve the participation of p21 in a direct upregulation of CREB targets other than MITF. In this regard, CREB protein activates several genes having a prosurvival activity, including bcl-2 and cox-2, and has been shown to act as a survival factor for melanoma cells (Jean et al., 1998; Xie et al., 1997). Previously, Kawata et al. (2003) observed that p21 enhanced the CREB-stimulated promoter activity and we showed that a synthetic promoter containing CRE sites is synergistically stimulated by forskolin and p21 in non-melanoma cells (Drdova and Vachtenheim, 2005), implicating p21 as a potential cofactor of CREB signaling. This was also supported by our present observation that p21 and CREB synergize in MITF promoter activation and CREB-directed shRNA partly reverted p21-mediated activation. Last, p21 protein might also contribute to melanoma cell proliferation through its known activity to promote formation of cyclin D-CDK4/6 complexes (Sherr and Roberts, 1999). Sequestration of p21 in these complexes liberates the Cdk2-associated kinase activity, which was described to be necessary for melanoma cell proliferation (Du et al., 2004).

Regardless of the precise biochemical mechanism by which p21 participates in MITF expression, our present data suggest a role for p21 as a transcription coactivator of MITF, resulting in a positive-feedback loop between MITF and p21 that may help to maintain the elevated MITF levels, and, consequently, increase the survival of melanoma cells.

Materials and methods

Cells and transfections

Melanoma cell lines used in this study have been described (Vachtenheim et al., 2001). Normal human melanocytes were purchased from Cascade Biologics (Invitrogen, Carlsbad, CA, USA) and maintained in the supplemented 254 medium as recommended by the manufacturer. B16-F10 mouse melanoma cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 4 mM glutamine, penicillin and streptomycin. For transfections, cells were seeded in a 24-well plate on the day prior to transfection. The following day the cells were transiently transfected with the appropriate amounts of reporter and expression plasmids using Lipofectamine 2000 reagent (Invitrogen). Luciferase assays were performed with the cell extracts obtained 48 h post-transfection by using the luciferase reagents obtained from Promega (Madison, WI, USA). Reference plasmids encoded either β-galactosidase or Renilla luciferase.

Plasmid constructs

Reporter constructs with the promoters of melastatin and tyrosinase, and the minimal promoter consisting of 5xGAL sites and E1b TATA box (5xGAL-Luc), were described previously (Drdova and Vachtenheim, 2005; Vachtenheim et al., 2007). Human melanocyte-specific MITF promoter contained nucleotides −2878 to +103 relative to the transcription start site cloned in the MluI-HindIII sites of pGL3-basic (Promega). In the CRE-mutated MITF promoter, the sequence TGACGTCA was changed to TGCA. TRP1-luc plasmid contained the −1411 to −68 bases of the human TRP1 gene cloned in pGL3-basic. The survivin promoter (nucleotides −941 to +39) was cloned in the same reporter plasmid. The GAL-p300(1-1303) expression vector encodes the first 1303 amino acids from human p300 cDNA linked to the GAL4 DNA binding domain. The expression plasmid pCMV-HDAC6 was obtained from Origene (Rockville, MD, USA); the pCMV-CREB and the pCMV-CREB133 (S133A mutant) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Expression constructs with the mouse p21 cDNA sequence, in which the amino acids 17–23 or 52–57 were deleted, were performed by PCR mutagenesis. The mouse p21(1-133)NLS construct contained only the first 133 amino acids of p21, followed by the nuclear localization signal from the SV40 large T antigen and a stop codon. The human p21(1-138)NLS construct was prepared similarly. Mouse p21 constructs were used for transfections of B16-F10 cells and human p21 constructs were transfected into human cell lines. All the p21 constructs were cloned in the plasmid with the FLAG-tag sequence at the amino-terminus (Sigma, St. Louis, MO, USA) and verified by sequencing.

shRNA constructs

The shRNA target sequences for the knockdown of human p21 (CGGCGGCAGACCAGCATGA) and MITF (GGACAATCACAACCTGATTG), and CREB (Dobroff et al., 2009) were cloned in the pSUPER.puro vector (Oligoengine, Seattle, WA, USA). The scrambled sequence (GACGGTACCGCGAACGAGC) was used for control transfections. For transient transfections, two additional p21 shRNA constructs (29-mers, constructs 1 and 4) were from Origene. To obtain clones of SK-MEL-5 cells with the stable knocked-down p21, cells transfected with the pSUPER.puro in which the p21 target sequence, or a scrambled sequence, was cloned, and cells were selected in 0.5 μg/ml puromycin for 2 weeks. Clones were analyzed for expression of the p21 protein by Western blotting. Several clones with greatly reduced p21 protein level were expanded and two of them used to determine the growth rate in triplicate wells.

Lentiviral infection

Sequences encoding both targeting and non-targeting shRNA were cut as EcoRI-ClaI fragments from pSUPER vectors and cloned into the same sites of the lentiviral vector pLVTHM (Wiznerowicz and Trono, 2003), obtained from Addgene (Cambridge, MA, USA). The lentiviruses were produced by transfecting human embryonic kidney cells 293FT (InVitrogen) with the appropriate shRNA cassette-containing pLVTHM plasmid (expressing also the green fluorescent protein, GFP), the envelope plasmid (pMD2.G), and the packaging plasmid (psPAX2). Supernatants containing the lentivirus were pooled, concentrated, and used for infections. Virus stocks were titered on H1299 cells. Cells were infected in the presence of 8 μg/ml Polybrene for 24 h, followed by the addition of a fresh medium, and cells were harvested for analysis 2 days later.

Immunoblotting

Whole cell extracts were prepared in RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate], containing 1 mM DTT, PhosStop (Roche, Mannheim, Germany), 1 mM AEBSF, aprotinin, leupetin and pepstatin. Protein levels were determined by Western blot analyzes by following established protocols using the ECL detection system (GE Healthcare, Uppsala, Sweden). The membranes were probed with primary antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), directed to p21 (C-19), cyclin E (C-19), cdk2 (M2), and GAPDH (V-8). Anti-MITF antibody (D5) was from Labvision

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(Fremont, CA, USA) and anti-FLAG antibody M2 was from Sigma. Secondary antibodies were goat anti-mouse or anti-rabbit IgG coupled to horseradish peroxidase (GE Healthcare). Western blot imaging and quantification of band intensity was performed with Kodak ID Image analysis software.

ChIP assays and real-time PCR analysis

Chromatin immunoprecipitation (ChIP) assays were carried out with the EZ ChIP kit (Upstate, Lake Placid, NY, USA). Formaldehyde-fixed cells were washed, lysed, and sonicated to shear the genomic DNA into fragments between 200 and 800 bp. Samples were precleared twice and immunoprecipitated with anti p21 rabbit polyclonal antibody (C-19) from Santa Cruz., anti-acetyl histone H4 antibody (Upstate) or control non-immune IgG. Antibody-containing complexes were recovered by adding the salmon sperm DNA-protein A agarose, washed as recommended by the supplier, and cross-links were reversed by heating the samples to 65°C overnight. DNA was purified and used for the PCR reaction.

Primer sequences for amplification of the MITF promoter region spanning the regulatory sites within the proximal promoter were 5′-TCGGCCATTGGAAAAGTCTC and 5′-TCCAGCATTTCCAGCATAACA. PCR conditions were 45 cycles, each for 25 s at 95, 62, and 72°C, using the Taq DNA polymerase (Roche). Control primers detecting the 166 bp fragment of the unrelated GAPDH gene were from Upstate.

Total RNA was extracted from melanoma cell lines using Trizol (Invitrogen) and cDNA was synthesized using oligo-dT primer and Superscript II reverse transcriptase (Invitrogen). To quantitate MITF transcripts, the primers and Taqman probe for real-time PCR was used as described by Dynek et al. (2008). The primers for human tyrosinase were 5′-CCAGAAGCTGACAGGAGATG and 5′-AGGCA TTGTGCATGCTGCTT. The probe for human tyrosinase was 5′-6-FAM-ACGGCGTAATCCTGGAAACCATGACA-TAMRA. Beta actin primers used in control amplifications were 5′-ATTGCCGACAG-GATGCAGAA and 5′-GCTGATCCACATCTGCTGGAA. The probe for β-actin was 5′-6-FAM-CAAGATCATTGCTCCTCCTGAGCGCA-TAM-RA. Quantitect Probe PCR Kit (Qiagen, Hilden, Germany) was employed for real-time PCR reactions as recommended by the supplier and the reaction products were detected with an ABI Prism 7700 instrument (Applied Biosystems, Foster City, CA, USA). Expression values for MITF were normalized to values of β-actin.

Acknowledgements

We thank Hana Novotna for excellent technical assistence. We also thank Dr. Evžen Křepela for help with real-time PCR experiments and image analysis of Western blots. We are grateful to Dr. Colin Goding for critical comments and suggestions and Dr. Jan Borovanský for continuous support and encouragement. This work was supported by grant no. NR/9319-3 from the Ministry of Health, Czech Rep.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Expression of the p53 protein in melanoma cell lines. Protein levels in whole-cell extracts were analyzed by immunoblotting with antibody against p53 (DO-1) and control anti-β-actin antibody.

Figure S2. Western blot analysis of MITF protein expression in melanoma cell lines. Protein levels in

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whole-cell extracts were analyzed by immunoblotting with antibody against MITF (D5) and control anti-GAPDH antibody.

Figure S3. Correlation of MITF and tyrosinase mRNA levels in human melanoma cell lines. Expression levels of mRNA in cell lines were estimated by real-time PCR method and standard errors from triplicate samples are shown. Analysis was performed in two separate experiments and one experiment is presented. Expression values were normalized to β-actin. The highest level of both parameters was set as 100.

Figure S4. Proliferation of SK-MEL-5 melanoma cell clones with decreased p21 protein. Clones expressing siRNA directed to p21 were selected and proliferation of two clones was analyzed. (A) Growth curves during the 4-day time course in culture. Each time point indicates mean of triplicate samples. The experiment was conducted two times with similar results; bars, ±SD. (B) Western blot confirming the lowered p21 protein level in shRNA-expressing clones. The level of β-actin was used as a control for protein loading.

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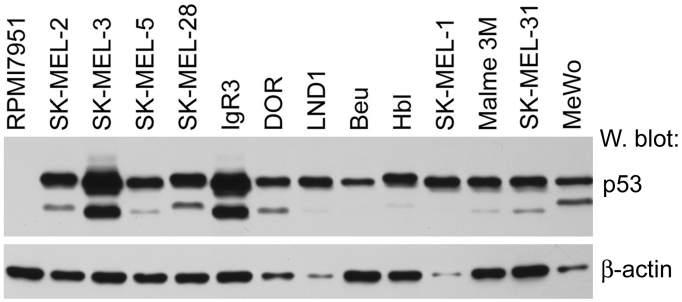

Figure S1 - revised

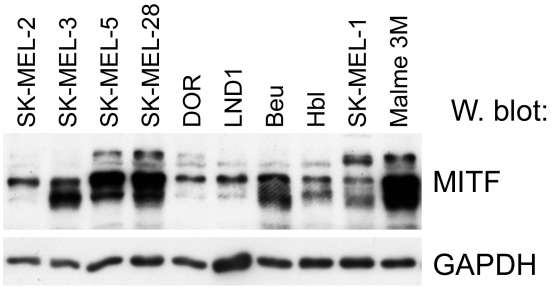

Figure S2 - revised

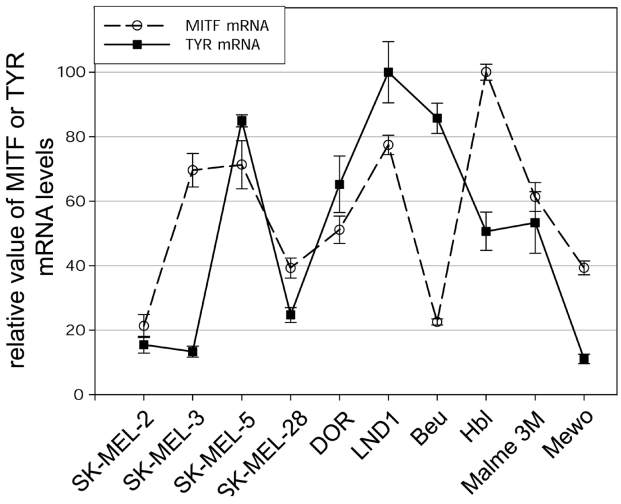

Figure S3 - revised

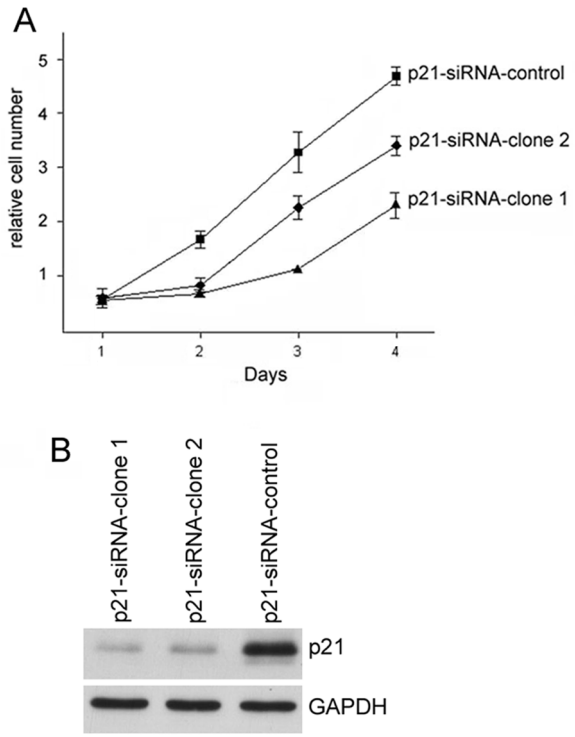

Figure S4 - revised

Inhibition of mTORC1 by SU6656, the selective Src kinase inhibitor, is not accompanied by activation of Akt/PKB signaling in melanoma cells

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Running Tittle:

Src activity controls the mTOR signaling in melanoma cells

Keywords:

mTORC1 signaling, Src activity, Akt/PKB, raptor, rictor, HBL melanoma cells

Footnotes:

The study was supported by grant NT11231-3/2010 from the Ministry of Health of the Czech Republic

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Abstract

The mammalian target of rapamycin (mTOR) is a Ser/Thr protein kinase conserved in all eukaryotes that plays a key role in cell growth and is a central effector of several pathways regulating essential cell functions. Hyperactivation of the mTOR-dependent signaling pathway occurs in many human diseases and may be a selective target for their therapy. However, the dual nature of mTOR, existing in two multiprotein complexes mTORC1 and mTORC2 driven by different feedback loops, decreases the therapeutic effects of rapamycin, the specific mTOR inhibitor. In the present study we demonstrate that the mTORC1 signaling pathway is highly activated in human melanoma cells and that upregulation of this pathway along with the growth and malignity of these cells could be suppressed by disruption of the Src activity. SU6656, the selective inhibitor of the Src kinase activity, decreased upregulation of the mTORC1 signaling and moreover, unlike rapamycin, it did not induce the activation of Akt/PKB and its downstream targets in HBL melanoma cells. The Src protein was found to be associated with raptor in the mTORC1 complex immunoprecipitated from these cells, suggesting that the Src activity might be a new attractive target for monotherapeutic inhibition of the upregulated mTORC1 signaling pathway.

Indroduction

Protein kinase cascades in cell signaling pathways are very often deregulated in human diseases and thus represent a valuable target for therapy. One of the most important protein kinase cascades that play a key role in many aspects of cellular physiology is the signaling pathway in which growth-regulating signals from outside and within the cell are integrated through serine/threonine protein kinase mTOR (mammalian Target Of Rapamycin). The mTOR signaling network is also influenced by nutrient and energy levels and is implicated in the regulation of transcription, autophagy, ribosome biogenesis, mRNA turnover, cytoskeletal organization and selective mRNA translation (Wullschleger et al., 2006). mTOR activation occurs downstream of PI3K-Akt signaling in this pathway. mTOR is negatively regulated by suppressor protein tuberous sclerosis complex (TSC1/2) comprising hamartin (TSC1) and tuberin (TSC2). Tuberin is directly phosphorylated by Akt/PKB, which disrupts the TSC1/2 complex and stimulates mTOR activity. mTOR exists in two multiprotein heterometric complexes, mTORC1 and mTORC2. More intensively studied signaling complex mTORC1 controls selective translation of mRNA (Ma and Blenis, 2009) and consists specifically of mTOR, Gβl and Raptor (Regulatory Associated Protein of TOR). The mTORC2 complex has been shown to particularly contain mTOR, Gβl and Rictor (rapamycin-insensitive companion of mTOR) and controls actin cytoskeleton dynamics (Loewith et al., 2002).

Constitutive activation of the Akt/mTOR pathway in cancer cells supports mTOR as a selective target for cancer therapy, with direct clinical applications. mTORC1 seems to be the only known target of the specific inhibitor of mTOR, drug rapamycin. Treatment with rapamycin inhibits proliferation and may lead to apoptosis, and thus rapamycin and its analogs (rapalogs) are evaluated in clinical trials as important anti-cancer agents. However, rapamycin has been found to enhance Akt/PKB activation in many cell lines, paradoxically activating signaling that reduced the anti-tumor effects of mTOR inhibition. Akt/PKB activation indicates the existence of a basal mTORC1-dependent feedback mechanism, probably induced by allosteric interactions of rapamycin with mTOR. In this feedback loop, mTOR associated with rictor in the mTORC2 complex directly phosphorylates Akt on serine 473, thereby contributing to Akt activation (Sarbassov et al., 2005). To avoid the events attenuating growth-inhibitory effects of rapamycin, combination therapies with dual inhibitors are in trials (Mallon et al., 2011). Also, a second generation of inhibitors has been recently developed that competitively target the ATP-binding site of the mTOR kinase domain to

block the mTOR protein kinase activity in both mTORC1 and mTORC2 simultaneously, though unsufficiently (Vilar et al., 2011).

We have previously demonstrated that the activity of non-receptor tyrosine kinase Src is involved in the upregulation of the mTOR signaling pathway (Vojtěchová et al., 2008) in RSV-transformed hamster fibroblasts and is suppressed by SU6656, the specific small-molecular inhibitor of Src kinase activity (Blake et al., 2000). In this study we used the inhibitor SU6656 to analyze the regulatory role of Src in the mTOR-dependent signaling pathway in melanoma cells. We found that the disruption of Src kinase activity by treatment of HBL melanoma cells with SU6656 suppressed mTORC1 signaling in a similar way as rapamycin and moreover, unlike rapamycin, treatment of the cells with SU6656 did not induce activation of Akt and its downstream signaling.

Materials and Methods

All general reagents were from Sigma (St. Louis, MO) unless otherwise stated. Protein G-Sepharose and ECL Prime WB detection reagent were obtained from GE Healthcare Bio-Sciences (Uppsala, Sweden). All primary and secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Cell Culture and Preparation of Cell Extracts

Human melanoma cell lines used in this study were described previously (Vachtenheim et al., 2001). HBL cells were cultivated in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (FBS), antibiotics, and L-glutamine. Malme 3M, MeWo, SK-MEL-28, SK-MEL-5 cells were grown in Eagle's minimal essential medium containing 10% FBS, antibiotics, L-glutamine, sodium pyruvate and non-essential amino acids. Normal human melanocytes were purchased from Cascade Biologics (Invitrogen, Carlsbad, CA) and maintained in the supplemented 254 medium as recommended by the manufacturer. Cells were brought to quiescence by an overnight incubation with 2% fetal calf serum and then treated with the inhibitor rapamycin or SU6656 added to a final concentration of 20 nM and 5 μM (DMSO solution diluted in a medium, $1{:}10^6$), respectively, or DMSO carrier alone for time periods as indicated. The cell lysates were prepared in ice-cold extraction buffer [50 mM TRIS pH 7.4, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, 40 mM β-glycerophosphate, proteinase inhibitors aprotinin, leupetin and pepstatin (0.4 μg/ml each), 1 mM PMSF and PhosSTOP (Roche)]. Cell debris was pelleted by centrifugation at 20000 g for 10 minutes, and supernatants were assayed for protein concentration.

Immunoprecipitation of Proteins, Western Blot Analysis

For immunoprecititation analyses the cell lysates were prepared in the extraction buffer containing 0.3% CHAPS instead of 1% Triton X-100. Proteins were immunoprecipitated from the cell lysates on Protein G-Sepharose beads, and the resulting immunoprecipitates were used for SDS-PAGE followed by immunoblot analysis as described (Vojtěchová et al., 2008).

Soft Agar Assay

Approximately 5×10^4 cells were seeded in the upper layer containing 3 ml of DMEM medium, 0.45 % agar, 10 % FCS supplemented with DMSO or inhibitors in a final concentration of 5 μM SU6656 or 20 nM rapamycin. The media were refreshed every 3-4

days. The colonies were stained with p-indonitrotetrazolium violet, counted, and photographed (magnification, × 20) after 24 days.

Proliferation Rates

HBL melanoma cells were seeded in triplicate wells, grown for time periods as indicated, fixed and stained with 0.1% crystal violet. The dye was quantified.

Results

Melanoma is a highly malignant skin cancer with multiple genetic defects including activating mutations in the B*raf* kinase gene and alternations in signal transduction pathways regulating proliferation and apoptosis (Zheng et al., 2009). We have found that in several melanoma cell lines (MeWo, Malme 3M, SK-MEL-5, SK-MEL-28, HBL), in contrast to normal human melanocytes, the activity of the mTOR-dependent signaling pathway is highly elevated (not shown). To analyze mTOR signaling in more detail, in this study we used a wild type of melanoma cells, HBL human cells (Herraiz et al., 2012), to avoid any possible misinterpretation of the obtained results due to the effects of BRAF mutations. Interactions between mutant BRAF and Akt/PKB pathway were identified in a subset of melanoma cells harboring mutant BRAF V600E (Chen et al., 2012).

Elevated Akt/PKB signaling upon rapamycin treatment of melanoma cells

Rapamycin is a well-known specific inhibitor of mTORC1 signaling in many human cancer cells and also in melanoma cells. To examine the effects of rapamycin in HBL cells we analyzed changes in the phosphorylation level of key effector proteins of the mTORC1 signaling pathway, mTOR, S6 kinase (S6K1) and its specific target, ribosomal protein S6. Phosphorylation of Ser2448 at mTOR decreased in cells treated for 60 min with rapamycin to about 40 % of the total mTOR phosphorylation in untreated control HBL cells. Rapamycin-induced inhibition of mTORC1 downstream signaling was rapid as judged by the increased electrophoretic mobility of protein kinase S6K1 and by the decreased binding of phospho-specific antibodies to Ser235/236 at ribosomal protein S6, as seen in Figure 1A. Rapamycin treatment for 15 min decreased phosphorylation of S6 protein to 80 % of the control, untreated cells, and complete inhibition required 30-60 min. Thr1135 phosphorylation of rictor, a component of the mTORC2 complex which was shown to be another target of the mTORC1-dependent kinase S6K1 (Treins et al., 2010), was also strongly inhibited in HBL cells upon treatment of the cells with rapamycin (Fig. 1A).

The positive effect of rapamycin in the suppression of hyperactivity of the mTORC1 signaling pathway is very often associated with increased activity of Akt/PKB induced by prolonged rapamycin treatment, probably by a feedback mechanism involving mTORC2 (Hresko and Mueckler, 2005; Sarbassov et al., 2005). In HBL cells, rapamycin treatment for 1, 2 and 8 hrs indeed increased phosphorylation of the regulatory amino acid residue, Ser473, as well as of Thr308 in the Akt molecule, generating completely activated Akt. The increased

phosphorylation and thus activation of Akt was associated with increased phosphorylation of Akt substrates, tuberin and MAPK, as compared with control untreated HBL cells (Fig. 1B). The increase in their phosphorylation began after 60 min of rapamycin exposure and remained elevated after 2 hrs and 8 hrs treatment. Prolonged rapamycin treatment, however, did not enhance the mTORC1/S6K1 signaling. Suppression of the phosphorylation of mTOR, S6K1, S6 and rictor was not changed upon treatment of the cells with rapamycin for up to 20 hrs (not shown).

SU6656, the selective inhibitor of Src kinase activity, decreased mTORC1 and Akt/PKB signaling

We have previously identified an important implication of Src tyrosine kinase activity in the regulation of the mTOR signaling pathway in RSV-transformed hamster cells (Vojtěchová et al., 2008). Treatment of v-*src*-transformed cells with SU6656, the specific small-molecule inhibitor of the Src family kinase, resulted in the decreased phosphorylation of signaling proteins in the mTORC1-dependent pathway in v-*src*-transformed cells.

Using SU6656, we now examined a possible role of Src activity in the upregulated mTOR-dependent signaling in HBL melanoma cells. As seen in Figure 2A, phosphorylation of mTOR (Ser2448), S6K1, S6 protein (Ser235/236) and rictor (Thr1135) decreased upon treatment of HBL cells with SU6656 in a time-dependent manner, similarly as in the cells that were treated with rapamycin (Fig. 1A). Attenuation of rictor phosphorylation at Thr1135 also by SU6656 is in agreement with the presumed fact that this phosphorylation is catalyzed by S6K1 (Treins et al., 2010; Julien et al., 2010).

Surprisingly, inhibition of the mTOR signaling pathway by SU6656 was not accompanied by increased phosphorylation and thus activation of Akt and its downstream signaling as it was seen in HBL cells treated with rapamycin (Fig. 2B). Moreover, incubation of the cells with SU6656 strongly decreased phosphorylation of Akt at Ser473 and Thr308 compared to untreated cells, implying that Akt is a substrate for Src kinase activity (Jiang and Qiu, 2003; Vojtěchová et al., 2008).

Src protein is associated with raptor in mTORC1

To detect possible interactions of Src with the mTOR complexes, we separated raptor, the adaptor protein of mTORC1, and rictor, the adaptor protein of mTORC2, from HBL cells by immunoprecipitation. As seen in Figure 3A, Src was present only in the immunoprecipitates of raptor, and not in the rictor immunoprecipitates. The association of Src with raptor agrees

with the presence of raptor only in the immunoprecipitates of Src from HBL melanoma cells (Fig. 3B).

Cell proliferation and soft agar cell colony formation are suppressed by both SU6656 and rapamycin

Exposure of HBL cells to rapamycin and SU6656 caused inhibition of cell proliferation at concentrations 5 μM SU6656 and 20 nM rapamycin, which are sufficient to profoundly inhibit mTORC1 signaling. The growth inhibition of HBL cells by SU6656 was slightly more effective than by rapamycin (Fig. 4A).

The soft agar assay was used to examine the capability of SU6656 and rapamycin to influence the phenotype of HBL cells. The block of cell colony formation correlates well with the inhibition of mTORC1 signaling induced by treatment of HBL cells with these inhibitors, indicating relations between malignant growth of cells and the mTORC1 activation. Rapamycin was again less efficient than SU6656 in suppressing malignant transformation of HBL melanoma cells, as shown in Figure 4B.

Discussion

Elevated tyrosine kinase activity of the protein product of c-*src* protooncogene was found in a number of human tumors and thus Src inhibition has recently become a target for drug therapy. Increased Src expression and activity exists in melanoma cell lines and in melanoma tumors *in vivo* (A. Barnekow et al., 1987; Homsi et al., 2007). It was shown that Src regulates STAT3, STAT5, βFGF receptors, N-cadherin and β-catenin in melanoma, and their activated forms were detected in both primary and metastatic melanoma in humans. Several melanoma cell lines were exposed to inhibitors of Src tyrosine kinase activity alone or in combination with standard chemotherapy agents (Homsi et al., 2009; Ferguson et al., 2013), although the specificity of these inhibitors was rather wide. Dasatinib, a multi-target tyrosine kinase inhibitor, was the first Src inhibitor approved for use in the treatment of adults with chronic myeloid leukemia (Eustace et al., 2008). However, the regulatory mechanism of Src is not quite clear.

As it is demonstrated in this paper, disruption of Src kinase activity in HBL melanoma cells by their treatment with SU6656, the selective inhibitor of Src kinase activity, decreased hyperactivity of the mTORC1 signaling in a similar way as the treatment with specific inhibitor of mTOR, rapamycin. In addition, inhibition of mTORC1 by SU6656 did not cause negative antiproliferative effects resulting from the activation of Akt/PKB signaling by rapamycin treatment, suggesting that suppression of Src activity may block this feedback mechanism. On the other hand, allosteric changes in the mTOR molecule induced by the inhibitory binding of FKBP 12-rapamycin complex to mTOR probably could result in Akt/PKB phosphorylation by mTORC2.

The presence of Src in the immunoprecipitates of raptor observed in this study indicates that Src may be bound to raptor and that changes in the Src tyrosine kinase activity may thus directly modulate the function of raptor to serve as a scaffold protein of mTOR in mTORC1. There are two key regulatory proteins in the multiprotein mTORC1 and mTORC2 complexes, raptor (Kim et al., 2002) and rictor (Dibble et al., 2009). They are essential for the specific function of each complex, acting as adaptor proteins phosphorylated in multiple sites. This implies that raptor and rictor may be modified by different kinases integrating different signals and playing an important role in the control of mTOR signaling.

Rictor was found to also be a direct target of rapamycin-sensitive complex mTORC1, being phosphorylated by S6K1 at Thr1135 and controlled by the mTORC1 activity. In this study, rictor phosphorylation at Thr1135 was inhibited in HBL cells that were treated by both

rapamycin and SU6656. These data are in accordance with the findings that placed rictor downstream to S6K1, indicating a functional relationship between mTORC1 and mTORC2, and support the possibility that rictor may not be required for rapamycin-induced phosphorylation of Akt/PKB by mTORC2 (Boulbes, et al., 2010).

We have reported previously that in v-*src*-transformed cells, the Src kinase activity is essential for the activity of mTOR-dependent pathway in cells expressing hyperactive Src protein, and we suggested that the mTORC1 signaling may be controlled by Src independently of Akt/PKB (Vojtěchová et al., 2008). The fact that basal phosphorylation of Akt/PKB was profoundly decreased by SU6656 treatment suggests simultaneous effects of separate Src inhibition on the activity of Akt/PKB (Jiang and Qiu 2003, Vojtěchová et al., 2008) and mTORC1 activity, probably preventing activation of Akt/PKB, which can be induced by the rapamycin-mTOR interactions.

Increasing evidence supports mTOR as a selective stable genetic target for direct therapy with clinical applications in human diseases. Data presented here suggest that specific inhibitors of the Src kinase activity might be promising monotherapeutic agents for inhibition of the mTORC1 signaling in melanoma cells and may lead to improvements in the therapy of this troublesome cancer.

Acknowledgements

The study was supported by grant NT11231-3/2010 from the Ministry of Health of the Czech Republic to Z.T. L.O. participates in the Ph.D. program at Charles University in Prague, Third Faculty of Medicine, Charles University in Prague, Czech Republic

Figure Legends

Fig. 1

Phosphorylation of (A) mTOR, S6K1, ribosomal protein S6, rictor, and (B) Akt, tuberin, MAPK in HBL melanoma cells that were incubated with rapamycin.

Crude extracts (15 µg) of HBL melanoma cells that were incubated with rapamycin (20 nM) (+) or with vehicle, DMSO (-), for periods of time as indicated were analyzed by Western blotting. Phosphorylation of mTOR, S6 protein, rictor, Akt, tuberin, and MAPK was examined using specific antibodies against phosphorylated proteins. The phosphorylation state of S6K1 is demonstrated by the gel shift method, where the highest phosphorylated form of S6K1 moves in the gel more slowly (γ, δ) than the less phosphorylated (β) or unphosphorylated (α) form of S6K1, as described in Materials and Methods. Numbers at the bands express their densitometric evaluation related to their relevant control bands that are taken as 100. Data are the means of at least three independent experiments from which one experiment is depicted.

Fig. 2

Phosphorylation of (A) mTOR, S6K1, ribosomal protein S6, rictor, and (B) Akt, tuberin, MAPK in HBL melanoma cells that were incubated with the Src inhibitor SU6656.

Crude extracts (15 µg) of melanoma cells HBL that were incubated with the inhibitor SU66S6 (5 µM) (+) or with vehicle, DMSO (-), for periods of time as indicated were analyzed by Western blotting. Phosphorylation of mTOR, S6 protein, rictor, Akt, tuberin, and MAPK was examined using specific antibodies against phosphorylated proteins and the phosphorylation state of S6K1 is demonstrated by the gel shift method as described in Fig. 1. Numbers at the bands express their densitometric evaluation related to their relevant control bands that are taken as 100. Data are the means of at least three independent experiments from which one experiment is depicted.

Fig. 3

The presence of Src protein in the raptor complex in HBL melanoma cells.

Total cell lysate of HBL melanoma cells (15 µg), (**A**) raptor and rictor complex immunoprecipitated from the cell lysates, and (**B**) Src protein immunoprecipitated from the cell lysate (400 µg) were analyzed b**y** Western blotting using specific anti-raptor, anti-rictor

and anti-Src antibodies as described in Materials and Methods. Two of five independent experiments are depicted.

Fig. 4

Proliferation rates (A) and soft agar colony formation assay (B) of HBL melanoma cells. (A) HBL melanoma cells were grown in the presence of DMSO (●), 5 μM SU6656 (□) or 20 nM rapamycin (◊) for the time periods as indicated. The relative cell number was estimated by staining as described in Materials and Methods. The values are means of duplicates ± SE. (B) The soft agar assay of the effect of the inhibitors, rapamycin and SU6656, on the malignant properties of HBL melanoma cells was carried out in 60 mm Petri dishes as described in Materials and Methods.

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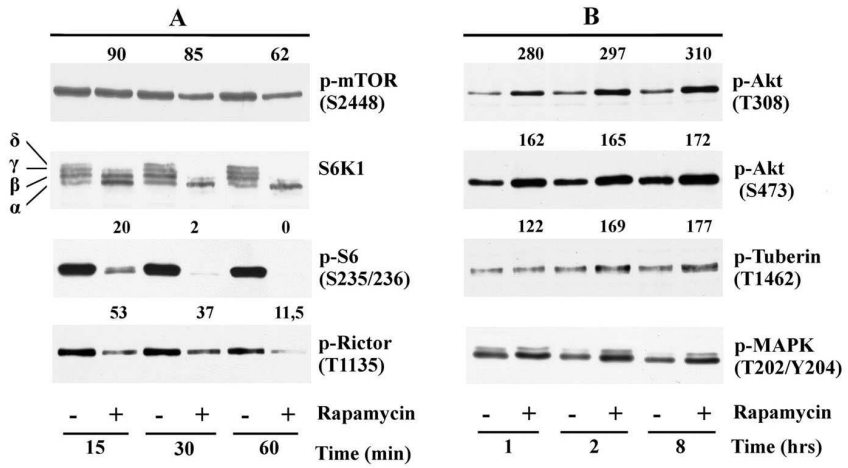

Fig. 1

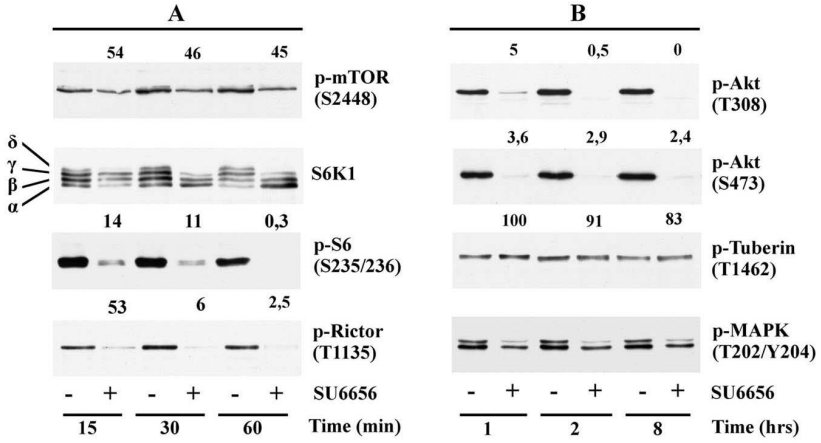

Fig. 2

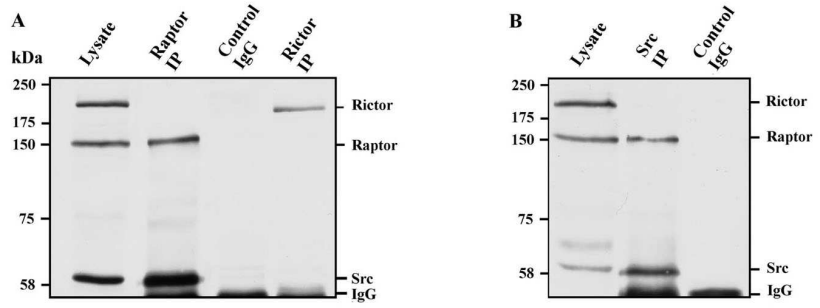

Fig. 3

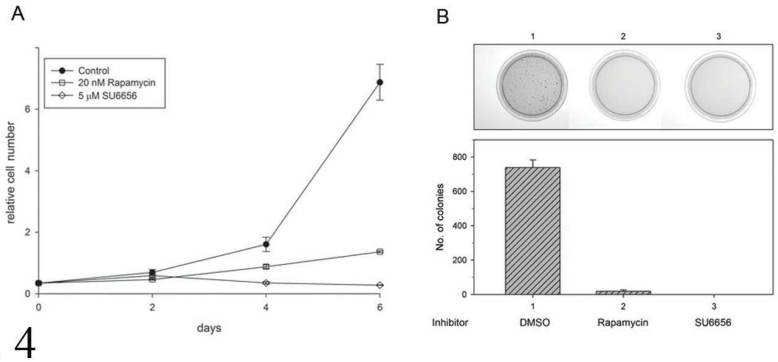

Fig. 4

Src-dependent activation of the mTOR signaling in melanoma cells

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SUMMARY

The mammalian target of rapamycin (mTOR) is an important signaling protein that integrates external and internal signals to regulate essential cell functions in a variety of organisms. Deregulation of the mTOR-dependent pathway occurs in many human diseases and may be a selective target for their therapy. However, clinical results obtained by targeting this pathway with mTOR-specific inhibitor rapamycin have shown its limited therapeutic effects. We have found that mTOR signaling is highly upregulated in melanoma cells. This hyperactivity could be reduced by treatment of the cells with rapamycin and by suppression of the activity of tyrosine kinase c-Src, which is comparable with the effect of rapamycin. Inhibition by the Src inhibitor does not lead to phosphorylation of Akt that can be induced by rapamycin, probably by a feedback mechanism.

INTRODUCTION

Several signaling pathways are deregulated in human diseases and represent a valuable target for therapy. One of the most important cellular pathways is the signaling pathway in which growth-regulating signals from within and outside the cell are integrated through serine/threonine protein kinase mTOR (mammalian Target Of Rapamycin). This kinase exists in two heterometric complexes: mTORC1 and mTORC2. One of important negative regulators of mTOR is the suppressor protein tuberous sclerosis complex (TSC1/2) comprising hamartin (TSC1) and tuberin (TSC2). Tuberin acts downstream of phosphatidylinositol 3-kinase (PI-3K) and protein kinase B (Akt/PKB), and is directly phosphorylated and thereby regulated by Akt/PKB. Phosphorylation of tuberin disrupts TSC1/2 and stimulates the activity of mTOR. mTORC1 is involved in the control of translation and is the target of mTOR-specific inhibitor, drug rapamycin. The mTORC2 complex controls actin cytoskeleton dynamics and is probably involved in a negative feedback mechanism [1].

More intensive studies have been focused on the complex mTORC1, whose signal regulates selective translation of the specific subsets of mRNA. An important target of mTORC1 is p70 S6K (S6K1). The activity of this protein kinase and the resulting phosphorylation of its physiological substrate, ribosomal S6 protein, is involved in translational control of a subset of 5'TOP mRNAs. Many of these mRNAs code for critical proteins regulating cell growth, cell proliferation, and cell cycle progression [2]. The data in this study show that in HBL and SK-MEL-5 melanoma cells, the mTORC1 signaling pathway is highly activated. This hyperactivity can be efficiently reduced by treatment of the cells not only with rapamycin, but also with SU6656, a small molecule inhibitor of Src kinase activity.

MATERIALS and METHODS

All general reagents were from Sigma (St. Louis, MO) unless otherwise specified. All antibodies were from Cell Signaling Biotechnology (USA).

Cell culture. Human melanoma cell lines have been described previously [3]. HBL cells were cultivated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), antibiotics, and L-glutamine. SK-MEL-5 cells were cultivated in Eagle's minimal essential medium containing 10% FBS, antibiotics, L-glutamine, sodium pyruvate and non-essential amino acids. Cells were brought to quiescence by overnight incubation with 2% FBS and then treated with inhibitor SU6656 or rapamycin added to a final concentration of 5 μM and 20 nM (DMSO solution diluted in medium $1:10^6$), respectively, or DMSO carrier alone.

Preparation of cell extracts and immunoblotting. The cells were extracted in lysis buffer (50 mM TRIS (pH 7.5), 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, aprotinin, leupetin, pepstatin (0.4 μg/ml each), 1 mM PMSF and PhosStop). Cell debris was pelleted by centrifugation at 20 000 *g* for 10 min and supernatants were assayed for protein concentration. Fifteen μg of protein lysate was subjected to SDS-PAGE/immunoblot analysis as described [4]. Experiments were repeated at least three times and quantified using densitometry.

RESULTS

As demonstrated in **Fig. 1**, highly increased phosphorylation of the ribosomal protein S6 on serine 235/236 and phosphorylation of Akt on threonine 308 was observed in melanoma cell lines SK-MEL-5 and HBL, but not in normal melanocytes. This indicates that the activity of the mTORC1-dependent signaling pathway is upregulated in these malignant melanoma cells.

Phosphorylation of both effector proteins, S6 and Akt, was decreased upon treatment of the cells with either rapamycin or the inhibitor of Src kinase activity, SU6656 [5]. The inhibitory effect of rapamycin (**Fig. 2A**) on the phosphorylation of mTOR on serine 2448 and of ribosomal protein S6 (Ser235/236) in HBL melanoma cells is time dependent, similarly as is the effect of treatment with SU6656 on the phosphorylation of mTOR and S6 protein of these cells, as seen in **Fig. 2B**.

Although rapamycin is a specific and efficient inhibitor of mTORC1 and its downstream targets, the inhibition of mTOR by rapamycin was also shown to induce a negative feedback mechanism which can be responsible for the phosphorylation and thus activation of Akt in some cancer cells [6]. In HBL melanoma cells, phosphorylation of Akt on threonine 308 increased 2.5-fold upon treatment of cells with rapamycin for 2 hrs and app. 3-fold upon treatment for 8 hrs compared to the phosphorylation status of Akt (Thr308) in the control, untreated cells (**Fig. 3A**). Conversely, as seen in **Fig. 3B**, phosphorylation of Akt on threonine 308 was completely suppressed upon treatment of HBL cells with the inhibitor of Src kinase activity, SU6656, for the same time period.

CONCLUSIONS

Increasing evidence supports mTOR as a selective stable genetic target for therapy in human diseases. Rapamycin treatment inhibits proliferation and may lead to apoptosis, and rapamycin analogs are evaluated in clinical trials as important anti-cancer agents. Although

this drug is a selective inhibitor of mTORC1 and its downstream signaling, the inhibition of mTORC1 by rapamycin was also shown to induce activation of PI3K/Akt signaling pathway. These events seem to attenuate the growth-inhibitory effects of rapamycin in the therapy by a negative feedback mechanism which could induce drug resistance. As shown by our findings, suppression of the Src activity by SU6656 may lead to profound inhibition of mTOR signaling without activation of the endogenous Akt in melanoma cells. Thus, the data presented here suggest that inhibitors of the Src kinase activity might be promising agents for inhibition of mTORC1 signaling in some types of cells.

Aknowledgement
The study was supported by grant NT11231-3/2010 from the Ministry of Health of the Czech Republic

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FIGURE CAPTIONS

Fig. 1

Western blots of the crude cell extracts (15 μg) of melanocytes and SK-MEL-5 and HBL melanoma cells. Phosphorylation of ribosomal protein S6 (p-S6) and Akt (p-Akt) was analyzed using specific anti-phosphoSer235/236-S6 antibody, and anti-phosphoThr308-Akt antibody. The cells were either untreated (-), or treated with rapamycin (R) or SU6656 (SU) for 60 min. Total amounts of Akt in the cells were examined with specific anti-Akt antibody.

Fig. 2

Phosphorylation of mTOR and S6 ribosomal protein in melanoma cells HBL that were incubated either with rapamycin (A) or with the Src inhibitor SU6656 (B). Crude extracts (15 μg) of melanoma cells HBL that were incubated with rapamycin (20 nM) (+) or with vehicle, DMSO (-) (**A**), and with inhibitor SU66S6 (5 μM) (+) or with vehicle, DMSO (-) (**B**) for the indicated period of time were analyzed by Western blotting using specific antibodies against phosphorylated proteins, the anti-phosphoSer2448-mTOR antibody and anti-phosphoSer235/236-S6 antibody.

Fig. 3

Phosphorylation status of Akt in melanoma cells HBL that were incubated with either rapamycin (A) or Src inhibitor SU6656 (B). Crude extracts (15 μg) of melanoma cells HBL that were incubated with rapamycin (20 nM) (+) or with vehicle, DMSO (-) (**A)** and with inhibitor SU66S6 (5 μM) (+) or with vehicle, DMSO (-) (**B**) for the indicated period of time were analyzed by Western blotting. Phosphorylation of Akt was examined using specific anti-phosphoThr308-Akt antibody. (**C**) Densitometric evaluation data of the bands represent the means of at least three experiments.

Fig. 1

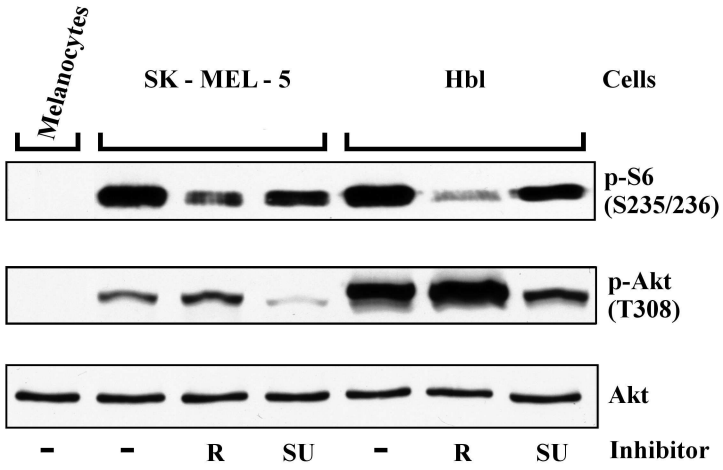


Fig. 2

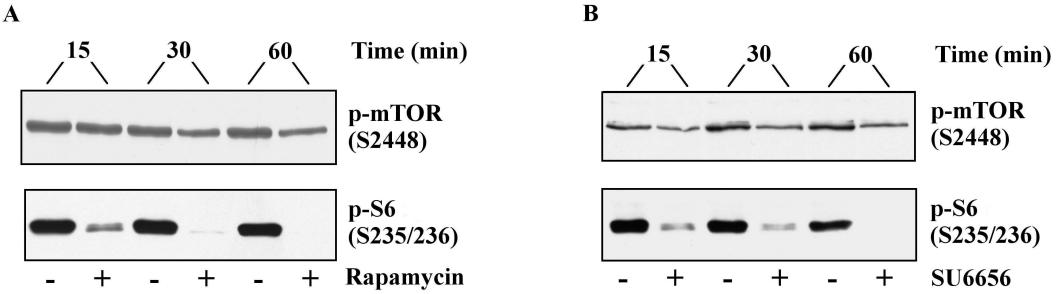
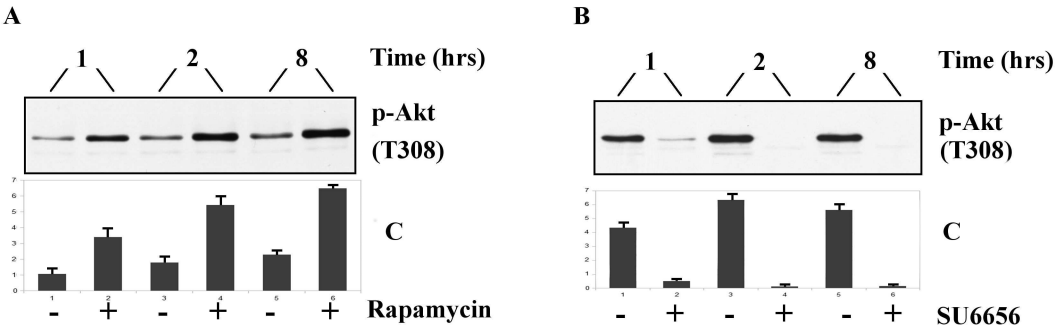


Fig. 3



In: New Developments in Chromatin Research
Editors: Neil M. Simpson and Valerie J. Stewart
ISBN: 978-1-62081-816-9
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Chapter 5

MITF MEETS CHROMATIN IN MELANOMA

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ABSTRACT

SWI/SNF chromatin remodeling complexes contain either Brm (Brahma) or Brg1 (Brahma-related gene 1) as the ATPase subunits. These ATP-dependent complexes are specialized protein machineries which remodel the nucleosomes to make the DNA accessible during processes like transcription, DNA repair and DNA replication. MITF (microphthalmia-associated transcription factor) is a pivotal protein required for the survival and proliferation of normal melanocytes and melanoma cells. MITF activates transcription of many genes regulating proliferation, apoptosis and invasiveness of melanoma cells and is a potential target for gene therapy of melanoma, an aggressive malignant tumor for which no effective therapy exists when metastases occur. It has been shown that many MITF target genes require SWI/SNF remodeling complex for expression. Importantly, expression of MITF itself is absolutely dependent on this complex, and both Brg1- or Brm-containing complexes are sufficient to support MITF gene transcription. Additionally, especially Brg1 complex seems to support tumor growth even independently of the MITF axis. Thus, SWI/SNF complexes appear to be crucial determinants of melanoma development and progression.

Keywords: SWI/SNF, MITF, melanoma, Brg1, Brm, INI1

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INTRODUCTION

Melanoma is known for its aggressive biological and clinical behavior and resistance to conventional anticancer therapies (Miller and Mihm, 2006; Gray-Schopfer et al., 2007). Although melanoma may have various rare locations, it is predominantly localized in the skin. It originates from melanocytes, specialized pigmented cells that are found mainly in the skin and eyes, where they produce pigments melanins, which are responsible for skin and hair colour. In the skin, melanocytes normally reside in the basal layer of the epidermis surrounded by skin keratinocytes and in the hair follicles. The benign precursors of skin melanoma are nevi, lesions composed of specific neval melanocytes which rarely develop to malignant tumor. However, studies postulate the presence of a specific nevus type, a dysplastic (atypical) nevus, as a factor associated with increased risk of developing skin melanoma. The risk factors for melanoma are a family history of melanoma, multiple benign or atypical nevi, and intermittent exposure to UV irradiation. Transformation of melanocytes and subsequent tumor progression from superficially spreading melanoma to metastatic disease are thought to be governed by a complex of changes involving the accumulation of genetic alterations and epigenetic changes in chromatin structure.

BRAF (V600E) is a predominant activating mutation in sporadic melanoma that has been identified ten years ago (Davies et al., 2002). About 60-70% of melanomas carry this mutation. However, mutated N-Ras was the first oncogene identified in melanoma in the 1980s and is present in around 10–25% of all tumors. The mutation in N-Ras is mainly a L61Q substitution. Either BRAF or N-Ras (acting through CRAF) mutation is found in tumor samples and both constitute a strong activation signal for the MEK/ERK pathway, which is invariantly deregulated in melanomas (Davies et al., 2002; Gray-Schopfer et al., 2007). Targeting mutated BRAF or mitogen-activated protein kinase (MAPK) pathway by small molecule inhibitors are therefore believed to be promising approaches to treatment of melanoma (Bollag et al., 2010). Interestingly, cells in benign nevi already contain BRAF (or N-Ras) mutation that confers senescence to these cells by a mechanism called oncogene-induced senescence, thus preventing malignant transformation (Michaloglou et al., 2005). Mainly p16(INK4A) tumor suppressor protein is thought to be responsible for the senescent status (Michaloglou et al., 2005), although other genes may be possibly involved because senescence does not strictly require p16 (or p14ARF) (Haferkamp et al., 2009; Yu et al., 2009) and expression of p16 is lost in a large proportion of benign nevi (Michaloglou et al., 2005; Scurr et al., 2011).

Thus, melanocytes require additional genetic changes (e.g., abrogation of the p16 function or inactivation of p53 or p14ARF) to bypass the senescent state and develop melanoma (Delmas et al., 2007; Yu et al., 2009). Mutation in the p16(INK4A) gene is a long known germline lesion predisposing to melanoma and occurring in a large portion of familial melanomas (Hughes-Davies, 1998). Loss of this protein may thus likely cooperate with BRAF mutation in the pathogenesis of primary melanomas arising from nevi (Hansson, 2010; Scurr et al., 2011). It should be noted that, besides p16, germline activating mutations in the CDK4 (cyclin-dependent kinase 4) might occur in the familial form of melanoma (Zuo et al., 1996). This review will focus on another aspect of melanoma progression, an epigenetic mechanism that is now becoming to appear to govern pivotal roles in melanoma cell transcription. We will describe the function of the mammalian SWI/SNF chromatin

remodeling complex in melanoma cells and suggest this complex as a possible target for gene therapy.

MITF and Melanoma

The MITF gene encodes a member of the large number of the basic helix-loop-helix zipper transcription factors. The small MITF subfamily of these factors (the MiT family) includes also related transcription factors TFEC, TFE3 and TFEB. MITF has many isoforms which differ in their N-termini because of the nine different promoters and differential splicing across the different first exons into a common second exon (Hou and Pavan, 2008). The M-isoform (named MITF-M, referred to as MITF throughout this chapter) is expressed exclusively in the melanocyte lineage. The melanocyte-specific promoter of MITF has been cloned (Fuse et al., 1996) and is functional exclusively only in pigmented cells. Of the many tissue-specific promoters of MITF, promoter for MITF-M is located most proximally to the first exon.

Several transcription factors were shown to activate MITF expression. These include Sox10, Pax3, Lef-1 and CREB (Goding, 2000; Vachtenheim and Borovansky, 2010). The β-catenin pathway, which has been reported to require SWI/SNF remodeling complex for the activation of target genes (Barker et al., 2001), is a prominent activator of MITF expression and its melanoma pro-proliferative activity is dependent on MITF (Widlund et al., 2002), and significantly contributes to melanoma progression and chemoresistance (Sinnberg et al., 2011).

The p300/CBP transcriptional coactivators are histone acetyltransferase enzymes with extensive structure homology (Kalkhoven, 2004) and high conservation between species. They were described to function as cofactors for expression of MITF downstream genes already before many years. P300/CBP interact with MITF in vitro and in cells and are believed to activate the transcription of MITF targets, possibly by acetylating the histones in nucleosomes within the target promoters (Sato et al., 1997; Price et al., 1998). Alternatively, acetylation of MITF itself by p300 and PCAF (p300/CBP-associated factor) might affect its transcriptional activities (our unpublished observation). Controversially, however, we blocked the cotranscriptional function of p300 with the N-terminus of the adenoviral E1A oncogene and did not achieve the repression of MITF activity. Interestingly, the E1A CR1 domain (which alone is insufficient to bind and block p300 function) was sufficient for repression (Vachtenheim et al., 2007). This observation that the p300-non-binding E1A mutants still activate an endogenous MITF target (tyrosinase evoked by transfected MITF in U-2 OS cells) weakens the necessity of p300/CBP as a MITF cofactor. Furthermore, the mutation of serine 73, the phosphorylation of which is required for p300 interaction at least after the Kit signaling (Hemesath et al., 1998), and the N-terminus truncated mutants (up to aa. 105) of MITF were fully capable of activating its responsive promoter-reporter and endogenous targets (Vachtenheim et al., 2007). These results do not necessarily exclude p300/CBP as co-activators of MITF but imply that possibly another cofactor different from p300/CBP might be important for MITF especially in the chromosomal context of the endogenous promoters.

In pigment cells, it is nevertheless believed that p300/CBP coactivators participate in the expression of MITF target genes and perhaps MITF itself. On the other hand, inhibitors of HDACs (histone deacetylases) like sodium butyrate, TSA, SAHA, and LBH589 were

effective in suppressing growth of melanoma cell lines and tumors in nude mice (Yokoyama et al., 2008). This was associated with transcriptional downregulation of the M-MITF promoter and MITF expression. Consequently, therapeutic applications either alone or in combination with other means of MITF suppression may have consequences for melanoma therapy. Inhibitors of HDAC have pleiotropic effects on cell metabolism and their effects cannot be simply explained as p300/CBP activators. First, HDACs can regulate activity of a number of non-histone proteins and second, histone acetylation regulates not only transcription, but also other fundamental processes such as DNA replication or mitosis. Inhibitors of HDACs are generally considered as promising drugs for cancer treatment, especially in combination with other agents (Lindemann et al., 2004; Rothhammer and Bosserhoff, 2007).

MITF regulates expression of a large set of pigmentation-specific genes (Goding, 2000; Vachtenheim and Borovansky, 2010) and genes promoting proliferation and invasion of melanoma. High expression of MITF has been associated with proliferation and survival of melanoma cells via its target genes Bcl-2, livin (ML-IAP), c-met, CDK2, and HIF1α (McGill et al., 2002; Du et al., 2004; Busca et al., 2005; McGill et al., 2006; Dynek et al., 2008). However, MITF upregulates also genes with opposite function which are cell cycle inhibitors (p21 nad p16) (Carreira et al., 2005; Loercher et al., 2005) and it is thought that their transregulation is functional mainly in normal melanocytes. Detailed information on MITF target genes and MITF posttranslational modifications are beyond the scope of this review and can be found elsewhere (Goding, 2000; Steingrimsson et al., 2004; Goding and Meyskens, 2006; Vachtenheim and Borovansky, 2010).

MITF does not regulate only pigmentation and melanoma proliferation-related genes. It has been demonstrated that MITF possibly regulates transcription of nine miRNAs, some of which have been previously associated with melanoma. The promoters of these miRNAs contain evolutionary conserved MITF-binding nucleosome-free E-box motifs (Ozsolak et al., 2008). Although more miRNAs were associated with melanoma biology, MITF is required for expression of the miRNA-211 and its expression is regulated coordinately with the MITF target gene melastatin (TRPM1) (miRNA-211 resides in the sixth intron of TRPM1). MiRNA-211 was demonstrated to be a direct negative regulator of the proinvasive gene KCNMA1 (Mazar et al., 2010). Thus, as TRPM1, miRNA-211 reduces growth and invasiveness of melanoma. MiRNA-148 upregulated MITF expression (Haflidadóttir et al., 2010), while miRNA-137 and miRNA-340 reduced the expression of MITF (Bemis et al., 2008; Goswami et al., 2010). Possibly other miRNAs may regulate MITF (Ozsolak et al., 2008; Haflidadóttir et al., 2010).

MITF is regarded as a melanoma oncogene because mutations and amplifications were found in patients´ samples (Garraway et al., 2005; Cronin et al., 2009). Moreover, BRAF mutation and p16 inactivation accompanied amplification of MITF in melanoma cell lines and ectopic MITF in conjunction with the BRAF(V600E) mutant transformed primary human melanocytes. Thus MITF can function as a melanoma oncogene (Garraway et al., 2005; Garraway and Sellers, 2006; Levy et al., 2006). In another study, two of twenty six mutations in samples of primary melanomas were identified in the MITF coding sequence whereas four out of 50 analyzed metastatic samples showed mutations (Cronin et al., 2009). Moreover, 6 mutations of 55 samples of the metastatic lesions were demonstrated in the SOX10 gene, an upstream positive regulator of MITF, taking together about 20% abnormalities in the MITF pathway in melanomas. It has been also demonstrated that combined repression of

BRAF(V600) and MITF by shRNAs inhibited melanoma growth even in cell lines that were more resistant to MITF downregulation alone, as melanoma lines may display differentially sensitive to MITF-M depletion (Kido et al., 2009).

Recently, novel E318K MITF mutation has been identified. This mutation is a germline allele variant (Yokoyama et al., 2011). E318K MITF encodes an allele that has impaired sumoylation and differentially regulates several MITF targets; specifically, it enhances MITF protein binding to the HIF1α promoter, a MITF known target, and increases its transcriptional activity (Bertolotto et al., 2011; Yokoyama et al., 2011). The presence of this allele constitutes more than fivefold risk factor for melanoma and renal cell carcinoma. Further, the mutant protein enhanced melanocytic and renal cell carcinoma clonogenicity, migration and invasion, further implicating this MITF variant as a cancer risk factor for these two cancers (Bertolotto et al., 2011). Concerning amplifications, Garraway et al. (2005) found that MITF amplification occured in 3 of 30 primary cutaneous melanomas (10%) and 7 of 32 (21%) metastatic tumours. No amplifications were observed in the ten benign nevi tested (Garraway et al., 2005). Four MITF genomic amplifications out of 50 metastatic melanoma samples were described in an independent observation (Cronin et al., 2009).

The SWI/SNF Complex

The chromatin remodeling complexes are multisubunit protein machines which are capable of changing the local structure of chromatin. The energy for remodeling is provided by ATP and each of the complexes shares an ATPase subunit. Generally, the complexes function in a broad range of basic cell processes that include mainly regulation of transcription, DNA replication and repair, and homologous recombination (Neely and Workman, 2002; Lusser and Kadonaga, 2003; Hota and Bartholomew, 2011). The SWI/SNF nucleosome remodeling complex (the switch in mating type/sucrose nonfermenting complex in yeast) is highly conserved throughout evolution from yeast to human and the mammalian SWI/SNF is best characterized among other remodeling complexes. The SWI/SNF is strongly implicated in human cancer, as several SWI/SNF subunits has been reported missing or strongly downregulated in tumor cell lines and tumor samples, and numerous experimental findings suggest that this complex functions as a tumor suppressor (reviewed by Klochendler-Yeivin et al., 2002; Roberts and Orkin, 2004; Reisman et al., 2009; Halliday et al., 2009; Wilson and Roberts, 2011). In the cell, there exist several types of SWI/SNF complexes, but principally the main two are predominant and are characterized by the presence of an ATPase subunit, which is either Brm (SMARCA2, brahma, BAF190B) or Brg1 (SMARCA4, brahma-related gene 1, BAF190A). The BAF subcomplex can be assembled with either Brm or Brg1, while PBAF subcomplex contains only Brg1. BAF complex contains BAF250A or BAF250B (ARID1A or B) as diagnostic subunits, while PBAF contains BAF180 (PBRM1, also known as polybromo) and BAF200 (ARID2, polybromo-associated factor) as specific components (reviewed by Reisman et al., 2009). Other subunits are called BAFs and are shared by both complexes; they include BAF47/INI1/SNF5 (SMARCB1), BAF53A (ACTL6A) or BAF53B (ACTL6A), BAF57 (SMARCE1), BAF60A (SMARCD1) (or BAF60B, SMARCD2), BAF155 (SMARCC1), BAF170 (SMARCC2), and actin (ACTB). SWI/SNF, as other complexes, utilizes the energy provided by ATP hydrolysis to disrupt histone-DNA contacts of nucleosomes and alter nucleosome positions on DNA, resulting in the alteration of spacing

of nucleosomes, repositioning or disassembling nucleosomes. SWI/SNF thus activates genes by epigenetic mechanisms involving remodeling the local nucleosomes. However, in some instances, the complex is also capable of repressing genes, and especially Brg1 can enter several repressive complexes (besides BAF and PBAF) which contain additional proteins, which are typical cellular transrepressors, e.g. HDACs, mSin3a, Rb, or REST (Trotter and Archer, 2008). The presence of particular ATPase in SWI/SNF may confer substrate specificity to the complex (Kadam and Emerson, 2003). Thus, Brg1 or Brm may have synergistic and antagonistic effects on the SWI/SNF complexes, depending on the tissue and promoter context. Both can have antagonistic effects on the regulation of some genes (Marshall et al., 2003; Xu et al., 2007; Shen et al., 2008; Flowers et al., 2009), but are also interchangeable in some instances (Strobeck et al., 2002; Xu et al., 2007).

SWI/SNF IN DIFFERENTIATION AND CANCER

Numerous reports implicate the SWI/SNF complex as a crucial determinant of differentiation of many cell lineages (Simone, 2006; de la Serna et al., 2006a). For example, complexes with specific composition (npBAF) operate in neural development (Yoo and Crabtree, 2009). Important role plays the SWI/SNF in muscle formation (de la Serna et al., 2001; Ohkawa et al., 2007). The Brg1 ATPase and the muscle-specific transcription regulator MyoD are required in early stages of myogenesis (in developing embryonic tissue), whereas myogenin and Brg1 are critical for the expression of late genes during terminal muscle differentiation (Ohkawa et al., 2007). Also cardiac muscle growth and differentiation need the Brg1 function (Hang et al., 2010). Furthermore, various aspects of differentiation and maintenance of neural stem cells, the astrocyte and oligodendrocyte differentiation and switch from neurogenesis to normal gliogenesis in mice embryos have been shown to require Brg1 (Matsumoto et al., 2006). In addition to the above mentioned tissues, the proper osteogenesis (Young et al., 2005), erythropoiesis (Griffin et al., 2008), development of T-cells (Chi et al., 2002) and adipocytes (Salma et al., 2004) were reported to require the SWI/SNF complex. It has been demonstrated that specialized complexes (esBAF), defined by the presence of Brg1, BAF155, and BAF60A, and the absence of Brm, BAF170, and BAF60C are necessary for self-renewal and pluripotency of mouse ES cells (Ho et al., 2009). As shown recently in several articles and described in this chapter, the SWI/SNF complex is also essential for the maintenance and progression of melanoma cells. It might be envisaged that it can provide a similar crucial role in the embryonic development of the melanocyte lineage as well.

Two components of SWI/SNF, Brg1 and INI1, are often mutated or deleted in human tumors and cell lines, and are therefore regarded as tumor suppressors. A core SWI/SNF subunit, INI1, is inactivated in aggressive pediatric renal rhabdoid tumors, rhabdomyosarcomas, and some brain tumors (Versteege et al., 1998). Brg1, Brm or both are downregulated or inactivated in cancer cell lines and tumor samples derived mostly from non small-cell lung cancer (NSCLC) (Reisman et al., 2003; Glaros et al., 2007), but a variety of other cell types carry mutations or loss of expression of one of these two ATPases (Decristofaro et al., 2001). Medina et al. (2008) reported that about 25% of lung cancer cell lines carried homozygous mutation in Brg1, most of which predicted truncated protein. Vast majority of these mutations were in NSCLC cells. About 50% of intraductal papillary

mucinous pancreatic neoplasms, precursors of adenocarcinoma of the pancreas, also showed low immunohistochemical staining of Brg1, indicating protein loss (Dal Molin et al., 2011). The notion that Brg1 is a tumor suppressor in lung tumorigenesis was further substantiated by the observation that lung-specific heterozygous conditional knockout of Brg1 in mice developed increased number of carcinogen ethyl carbamate induced lung tumors. However, biallelic inactivation of Brg1 could not initiate tumor, indicating that transformation de novo required one Brg1 allele. Remarkably, when Brg1 was inactivated in mice exposed to the carcinogen, the tumor development was potentiated and some cells that were devoid of Brg1 formed larger and faster proliferating tumors (Glaros et al., 2008). Contrasting to Brg1, Brm expression is inactivated by epigenetic mechanisms (Glaros et al., 2007; Reisman et al., 2009).

Surprisingly, some types of cancer cells (gastric, prostate, colorectal, melanoma) display increased Brg1 or other SWI/SNF components. In colorectal cancer, expression of Brg1, but not Brm, was frequently increased, by immunohistochemical staining, and Brg1 knockdown suppressed proliferation by downregulating PTEN with subsequent activation of the Akt pathway and increased cyclin D1 levels (Watanabe et al., 2011). Notably, BAF155 is subject to interaction and phosphorylation by activated Akt, which might prove to be one of the potentialy regulatory mechanisms of SWI/SNF by activated signaling pathways (Foster et al., 2006). Prostate cancer cells revealed increased Brg1 (Sun et al., 2007) and androgen receptor required SWI/SNF for the expression of at least two of its targets (Marshall et al., 2003). Moreover, another SWI/SNF subunit, BAF57, substantially increased the androgen receptor activity, thus implicating this factor as molecular target for prostate cancer (Link et al., 2008). In gastric cancer, loss or decrease of Brm expression was observed in a large portion of tumor samples (Yamamichi et al., 2007) whereas increased Brg1 level was also implicated in pathogenesis of this malignancy (Sentani et al., 2001).

There exist numerous impelling data suggesting that INI1 may have SWI/SNF independent function, and SWI/SNF complexes lacking INI1 might provide autonomous functions other than fully assembled INI1-containing complexes. First, spectrum of tumors arising in monoallelically mutant Brg1 or INI1 mice are different, even though both proteins are regular components of the SWI/SNF complex (reviewed by Reisman et al., 2009; Wilson and Roberts, 2011), However, biallelic knockout of either gene is similar and is embryonically lethal at the peri-implantation stage (Klochendler-Yeivin et al., 2000; Roberts et al., 2000; Bultman et al., 2000). Second, some genes are upregulated by Brg1 in the absence of INI1, suggesting that the integral SWI/SNF complex is not essential for transcription, at least for some genes. These genes include for example CSF1, SPARC, HP8, and ID3 (Liu et al., 2001; Doan et al., 2004). Moreover, the complex is surprisingly fully assembled without the presence of INI1 (Doan et al., 2004). Thus, some genes require INI1 for transcription while other do not, implying that the INI1-deficient SWI/SNF complex can have yet unknown, INI1-dispensable cellular function. Third, paradoxically, INI1-deficient cancer cell lines are dependent on residual Brg1 activity. Ablation of Brg1 in these cells prevents tumor formation and therefore presumably INI1-deficient cancers disrupt INI1-independent (but Brg1-dependent) targets which may be the mechanism by which tumor formation is blocked (Wang et al., 2009). In normal diploid cells, INI1 loss causes cell death, so it is similar to embryonic lethality. On the other hand, hereditary MRT (malignant rhabdoid tumors) tumors survive and proliferate under complete loss of INI1. Thus, it can be suggested from these results that some yet unknown targets of Brg1 may probably enable

cancer cells to survive upon INI1 loss. Fourth, although the INI1 protein alone is capable of inhibiting cell cycle arrest by downregulating expression of several cell cycle activating proteins in MRT cells, it requires intact Rb protein for this ability. On the contrary, functional INI1 is not needed for Rb protein-mediated cell cycle arrest in these cells (Versteege et al., 2002). Further results have shown that ectopic Rb protein requires Brg1 for cell cycle arrest in Rb-compromised cells (Zhang et al., 2000; Versteege et al., 2002), thus clearly discriminating the role of INI1 and Brg1 in Rb-mediated cell cycle blockade. All these data together suggest the nonsynergistic role of INI1 and Brg1 in cancer cell formation.

MITF and SWI/SNF in Melanoma

Results from recent years indicate that melanoma is a tumor in which the pivotal transcriptional regulator MITF requires the SWI/SNF complex for the expression of its downstream genes as well as for its own expression. These findings placed SWI/SNF together with MITF on the central crossroad in the melanoma transcriptional network, influencing the basic processes of melanoma biology.

Mouse fibroblasts are capable of expressing pigment specific genes after transfer of ectopic MITF. Mouse fibroblasts inducibly expressing dominant negative (DN) forms of Brg1 or Brm were previously constructed and demonstrated to inhibit, upon induction, the activation of genes that normally require SWI/SNF enzymes. Inhibition of pigment cell-specific genes expression was observed after introduction of exogenous MITF in these cells (de la Serna et al., 2006b). This indicates the importance of SWI/SNF for MITF-directed gene expression in this heterologous cell model. Several melanocyte-specific markers such as tyrosinase, TRP1, TRP2 (tyrosinase-related proteins 1 and 2), and Pmel17 were downregulated. Strikingly, the function of cell cycle inhibitors p21 and p16, which are also MITF targets, was not affected by DN Brg1 or Brm, suggesting that SWI/SNF selectively promotes only pigment cell-specific genes in this experimental model. The TRP1 promoter was shown to bind MITF and it has been shown that Brg1 was associated (by chromatin immunoprecipitation) with this promoter together with MITF. Brg1 and MITF coimmunoprecipitated, and Brg1 colocalized with MITF in the nucleus of B16 melanoma cells (de la Serna et al., 2006b). In the zebrafish model, Brg1-mutated embryos showed defects in the development of pigment cells and retina, the tissues expressing MITF. This work suggests that Brg1 is involved in neural crest induction, and consequently in the development of neurons, glia, and pigment cells (Eroglu et al., 2006).

We have previously described prominent epigenetic mechanism in the tumorigenesis of melanoma by demonstrating that the expression of MITF is absolutely dependent on the functional SWI/SNF complex (Vachtenheim et al., 2010). We have shown generally high expression of SWI/SNF protein components in melanoma cell lines and have found only one cell line, SK-MEL-5, with the loss of the Brg1 gene expression. Brm has been shown to be present in all melanoma cell lines tested (Vachtenheim et al., 2010). However, other authors observed very low Brm expression in two melanoma cell lines tested (Keenen et al., 2010). Together, the components of SWI/SNF are generally well expressed in malignant melanocytes and at least one ATPase is always present in melanoma cell lines.

Because MITF critically requires SWI/SNF for its expression, knockdown of Brg1, Brm, or both with specific shRNAs has been performed in several melanoma lines. Although the

proliferation and survival have been transiently compromised in Brg1 knockdowned cells, melanoma cells recovered and continued proliferation, presumably because of the persistent expression of Brm. However, sequential knockdown of both Brm and Brg1 in 501mel cells completely abolished proliferation (Vachtenheim et al., 2010). In SK-MEL-5 melanoma cells, which do not express Brg1, depletion of Brm alone was sufficient to abrogate MITF expression and cell proliferation. When both Brg1/Brm knockdown was performed simultaneously, the transfected or lentivirus-infected cells were grossly deteriorated and a small number of remaining viable cells presumably survived due to an incomplete block of expression, which occurred when common Brg1/Brm shRNA was used. In chromatin immunoprecipitation assays, both Brg1 and Brm were capable of binding to the melanocyte-specific promoter of MITF (Vachtenheim et al., 2010). Expectedly, the expression of tested MITF targets (melastatin, CDK2, and tyrosinase) were decreased, much more prominently in the Brg1 knockdown experiments than in the Brm knockdown (Vachtenheim et al., 2010). These results together showed that either Brg1 or Brm in the SWI/SNF complex was able to support MITF expression, albeit Brg1 seemed to be more important ATPase. It should be noted that SWI/SNF may function in the expression of MITF through the upstream transcription factor LEF1, the β-catenin pathway effector, because β-catenin interacts with Brg1 and requires it for promoting the target genes in β-catenin signaling (Barker et al., 2001). The β-catenin pathway is frequently deregulated in melanoma (Widlund et al., 2002; Delmas et al., 2007; Sinnberg et al., 2011).

The dependence of several MITF targets on the SWI/SNF complex was confirmed recently to exist also in melanoma cells. As with MITF expression, some of its target genes strictly required the functional SWI/SNF. Similarly as in the mouse model (de la Serna et al., 2006b), Brg1 was detected on the promoters of genes that encode enzymes needed for melanogenesis, like tyrosinase, TRP1, TRP2, and RAB27A, and also proapoptotic gene livin (ML-IAP), however Brg1 did not bind to CDK2, Bcl-2, or Tbx2 promoters (Keenen et al., 2010; Saladi et al., 2010a). Furthermore, re-introduction of Brg1 in SK-MEL-5 cells, which express only Brm, enhanced expression of melanocyte-specific MITF target genes and increased pigmentation. As in the mouse cells, Brg1 interacted with MITF and was required for MITF target gene expression in melanoma cells. Importantly, re-expression of Brg1 in SK-MEL-5 cells significantly increased resistance to cisplatin and cell survival. Since SWI/SNF is required for MITF expression (see above), it should be noted that some of the effects ascribed to SWI/SNF or Brg1 in this work (Keenen et al., 2010), may have been produced by the manipulation with MITF level, through Brg1. Together all the results show that Brg1 might be the first ATPase in the expression of MITF and its downstream targets but it can be quite readily substituted by Brm. Probably both ATPases can participate in the expression of MITF and its endogenous targets in melanoma cells. In any case, at least one functional SWI/SNF ATPase would be required for survival of melanoma cells.

A large microarray approach which examined the nucleosome positioning in promoters, combined with the data from the chromatin immunoprecipitation analyses, revealed that MITF predominantly binds nucleosome-free regions, supporting the model that the presence of nucleosomes is a limiting factor for the accessibility of MITF to the promoters (Ozsolak et al., 2007). These data are entirely consistent with the requisite need of SWI/SNF-mediated remodeling for many of the MITF-driven promoters.

Tumor samples from melanoma patients were also investigated for the expression of Brg1, Brm and INI1 by immunochemical analysis. INI1 staining was reduced in tumor samples and correlated with poor prognosis (Lin et al., 2010, see below). The expression of Brg1 was found increased in the majority of biopsies and was found even in the first stages of melanoma development (primary melanomas). Staining was higher in all stages of tumor development. A significant difference in Brg1 expression was observed even between dysplastic nevi or primary melanoma when compared with controls (Lin et al., 2010). Moreover, the authors performed knockdown of Brg1 in melanoma cell lines by means of siRNA and revealed significantly reduced cell proliferative ability. This finding was due to G1 phase block and downregulation of cyclin D1. The data are concordant with the above described results on cell lines and further implicate Brg1 in melanoma initiation and pathogenesis. However, Becker et al. (2009) described low Brg1 immunohistochemical staining of primary and metastatic melanomas while Brm protein level was decreased only in ~20% of cases. This is a singular observation in melanoma and is in the contrast with other results. Perhaps Brg1 might be downregulated at later stages of tumor progression. Brm expression was mostly preserved, so Brm might become a critical remaining ATPase maintaining the expression of MITF in melanoma cells in vivo when Brg1 is lost. These authors also found an association between p16 and Brg1 but the functional molecular connection is unclear as Brg1 is not required for p16/INK4a-induced cell cycle inhibition (Becker et al., 2009). In contrast to melanotic lesions, squamous cell carcinomas of the skin had lowered levels of both Brm and Brg1. Brm protein showed low levels of mRNA and Brg1 protein loss appeared to be a post-translational event. In contrast, possible squamous cell carcinoma precursor, actinic keratosis, revealed normal levels of both proteins. Brm and Brg1 may be therefore novel tumour suppressor genes for human non-melanoma skin cancer, as they are downregulated during tumor progression (Bock et al., 2011).

Expression of INI1, estimated by IHC, was shown downregulated during progression of melanoma (from nevus to metastatic lesions), and its low or negative expression correlated with poor patients prognosis as an independent prognostic factor. Moreover, INI1 knock-down in melanoma cells increased the resistance of tumor cells to chemotherapeutics like doxorubicin, etoposide, and camptothecin. The downregulation of INI1 expression reduced drug-induced apoptosis in melanoma cells, while cell cycle was not appreciably affected by INI1 loss (Lin et al., 2009). However, in view of the above results showing the importance of Brg1 in melanoma cell survival, this observation is not so surprising as the SWI/SNF complex, as mentioned above, may have dual function, depending on either presence or absence of INI1. In this connection, one could speculate that the effect of INI1 was due to lower repression effect of INI1 on GLI1 transcription factor, an effector of the Hedgehog pathway (Jagani et al., 2010), which can be a crucial mechanism for the proliferation of INI1-deficient human malignant rhabdoid tumors (MRTs). It remains to be proven if this mechanism is valid also for melanoma cells, for which the Hedhehog-GLI1 signaling is also pivotal and positively regulates the proliferation and survival of human melanomas (Stecca et al., 2007).

The senescence and apoptosis induced by BRAF(V600E) in normal melanocytes has been revealed to be mediated by IGFBP7 (insulin growth factor binding protein 7). It has been further shown that this secreted protein upregulates BNIPL (BCL2/adenovirus E1B 19kD interacting protein 2-like protein). The BNIPL transcription required the SWI/SNF complex with Brg1 and INI1 recruited to the BNIPL promoter after the stimulation by

recombinant IGFBP7 (Wajapeyee et al., 2008). Because expression of IGFBP7 is lost by promoter hypermethylation in BRAF(V600E) melanomas, the restoration of the pathway was achieved by addition of recombinant IGFBP7 to melanoma cells, after which increased expression of BAF47 allowed recruitment of Brg1 to the BNIPL promoter. Thus, SWI/SNF seems to be involved even in the basic BRAF-MEK-ERK axis important for melanoma progression. This also might be the reason why INI1 level is often decreased in melanomas (Lin et al., 2009), possibly allowing the constitutive activation of the MAPK pathway.

Brg1, Brm, or INI1 mutations were previously not analyzed in melanoma samples, so it is unknown whether such mutations can occur in any stage of melanoma. However, infrequent somatic single-allele Brm mutations were found in non-melanoma skin tumors (in one of ten squamous cell and two of six basal cell carcinoma samples) (Moloney et al., 2009), consisting of uniform G:C to T:A transversions which are typical mutations occurring following UV irradiation. The transversion resulted in a substitution of glutamine by lysine at codon 203 (Q203K) in the conserved region of exon 4 of the Brm gene. Also, a Q228P polymorphism in the Brm gene locus was identified in the non-melanoma lesions (Moloney et al., 2009). The Brm mutation is a rare observation because epigenetic silencing is responsible for Brm repression in tumors (Glaros et al., 2007; Yamamichi et al., 2007; Reisman et al., 2009; Gramling et al., 2011).

Another important function of Brg1 in melanoma progression may come from the scarce recent findings indicating that it might support transcription of genes outside the MITF axis. One such situation has been described above, showing the Brg1 participation in the expression of BNIPL gene in the BRAF pathway (Wajapeyee et al., 2008). It has been further found that Brg1 modulates the expression of a subset of extracellular matrix enzymes and adhesion proteins (Saladi et al., 2010b). Further, Brg1 level was increased in primary melanomas, advanced melanoma, and melanoma cell lines. Activation of the enzyme metalloproteinase (MMP) 2 expression contributed to the Brg1 induced increase in melanoma invasiveness. Brg1 also bound the MMP2 promoter, indicating a direct regulation by SWI/SNF. Although Brg1 activated some genes with the antiproliferative activities like E-cadherin, the overall effect of Brg1 in SK-MEL-5 cells was proinvasive (Saladi et al. 2010b; Keenen et al., 2010). Remarkably, it was referred that Brg1, together with p300 may be required to restrict p53 activity in wild-type p53 tumors (Naidu et al., 2009). Depletion of Brg1, but not Brm, led to the activation of endogenous wt p53 and cell senescence. Although not described in melanoma cells, this mechanism might also contribute to the prosurvival role of Brg1 in melanomas, which do not have mutated p53.

Interestingly, we performed cDNA microarray analysis of shRNA-mediated knockdown of Brg1 in 501mel melanoma cells (Ondrušová et al., submitted). Longer cultivation deteriorated these sh-Brg1 cells but the cells later recovered probably because Brm replaced lost Brg1 function, suggesting that Brm is the surrogate ATPase supporting MITF expression (Vachtenheim et al., 2010). We have studied changes shortly after the complete inhibition of Brg1, ensuring that the change in expression pattern was due exclusively to Brg1 loss. Besides the profound downregulation of MITF and its target genes, we found that among the strongly repressed genes were also insulin growth factor 1 (IGF1) and osteopontin (OPN) (Ondrušová et al., submitted). These proteins are known to have proliferative and proinvasive activity in many cancers, including melanoma (Zhou et al, 2005; Hilmi et al., 2008). Further, survivin, TGFβ2, and also SOX10, an upstream MITF regulator important for embryonic development of the melanocyte lineage, were also downregulated. Decrease of all these

proteins were verified by RT-PCR and W. blotting (Ondrušová et al., submitted). OPN promoter was recently shown to be a target of activated Hedgehog pathway (Das et al., 2009), a signaling activated in melanoma (Stecca et al., 2007). Our data thus provide another evidence that the prosurvival role of Brg1 can be provided, besides activating the MITF axis, also through MITF-independent mechanisms in melanoma.

CONCLUSION

In conlusion, there may exist principally two types of cancer cells. For most cancers, SWI/SNF complex is a clear tumor suppressor (malignant rhabdoid tumors, non small-cell lung cancer, non-melanoma skin cancer, pancreatic cancer, and many other tumors, reviewed by Wilson and Roberts, 2011). For other types (prostate cancer, gastric and colorectal cancer, and melanoma), the SWI/SNF complex, or at least some of its components (mostly Brg1), seems to be a tumor promoter, probably by activating essential genes required for proliferation, antiapoptotic activity, invasivity or other characteristics of cancer cells.

ACKNOWLEDGMENTS

Grant support: This work was supported by a grant NT/11229-3 (to J.V.) from the Ministry of Health (IGA, MH). L.O. participates in the PhD program at Charles University in Prague, Third Faculty of Medicine, Czech Rep.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Chapter 4

MITF: A Critical Transcription Factor in Melanoma Transcriptional Regulatory Network

Jiri Vachtenheim and Lubica Ondrušová

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/55191

1. Introduction

Melanocytes are specialized cells found predominantly in the skin and eyes that form the pigment melanin, a polymer composed of several types of subunits formed from L-3,4-dihydroxyphenylalanine. Melanin is deposited in subcellular particles called melanosomes. Cutaneous melanocytes originate from neural-crest progenitors that migrate to the skin during embryonic development. In the skin, melanocytes reside in the basal layer of the epidermis and are present also in hair follicles. Skin melanocytes form an epidermal unit composed of one melanocyte having long cellular processes and about 30 keratinocytes.

Melanocytes are found also in a benign nevus, a common lesion observed in the skin which is a precursor of malignant melanoma. In the nevus, the melanocytes (called nevocytes) are morphologically different and although nevi are benign and the melanocytes in them are senescent, they may develop, though rarely, into melanoma, a highly malignant tumor whose incidence is rising steadily in western countries. Smaller part of melanoma tumors (approximately 10-15%) occurs in families with hereditary predisposition (Hansson, 2010). Most familial melanomas harbour germline mutations in the CDKN2A gene encoding the p16INK4 protein, a cdk inhibitor. Much smaller number of hereditary cases has germline mutations resulting in amino acid substitutions in the p14ARF or CDK4 proteins. Sporadic cases of melanoma predominate and can be grouped into four clinical subtypes: acral lentiginous melanoma, nodular melanoma, lentigo maligna and superficial spreading melanoma (SSM), which is by far the most common form of melanoma and develop into vertical-growth phase (VGP) melanoma. Lentigo maligna and SSM are most frequently associated with intermittent UV exposure (Gray-Schopfer et al., 2007).

About 60-70% of melanomas harbour mutations of the B-RAF gene (Davies et al., 2002) (in the vast majority the mutation is V600E), while mutations of N-RAS are present in about 10–25%

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of all tumors. Activating N-RAS and B-RAF mutations demonstrate mutual exclusivity in melanomas. B-RAF mutations are present already in nevus cells, conferring senescence to these cells by a mechanism known as oncogene-induced senescence, possibly explaining the low rate of malignant transformation of nevi. Activation of these two oncogenes triggers the MEK-ERK kinase cascade that is almost invariantly activated in melanomas; however, other signaling pathways can probably activate the cascade. Therefore, small molecule inhibitors of kinases in the pathway are considered as a promising tool for melanoma treatment (Solit et al., 2006; Bogenrieder and Herlyn, 2011). Although B-RAF inhibitors seem to be rational antimelanoma drugs, resistance occurs in a large proportion of patients, because other stimuli may also activate MAPKs (Alcala and Flaherty, 2012). Many other genes are deregulated during melanoma progression, mainly antiapoptotic genes and genes which are involved in the invasiveness, migration, and metastasis of melanoma, many of which are regulated by MITF-M (microphthalmia-associated transcription factor), a pivotal transcription factor of the melanocyte lineage (Figure 1).

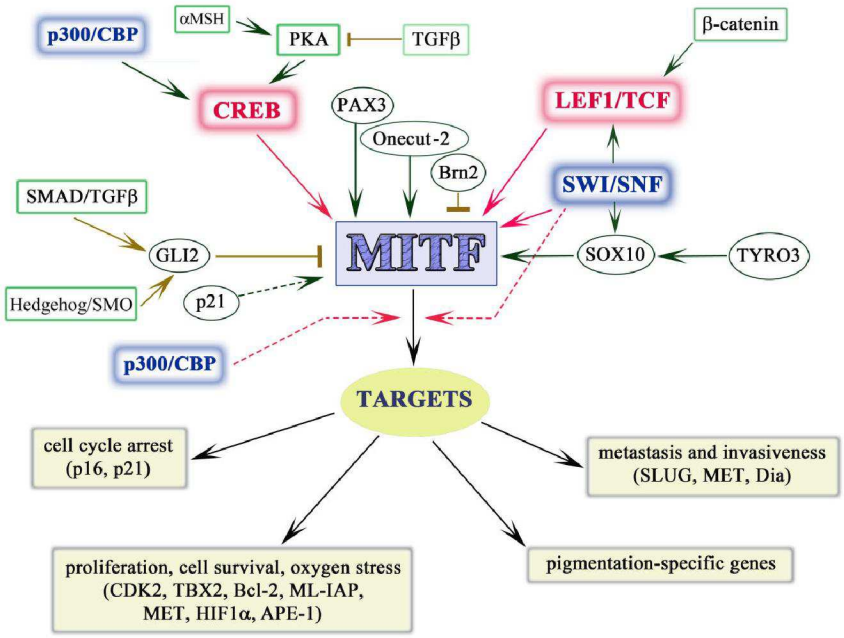

Figure 1. Transcriptional network of MITF-M in melanoma. The picture shows upstream MITF-M activators and main downstream transcription targets. The blue boxes indicate transcriptional coactivators and red boxes denote two transcription factors probably having more important role for the MITF-M expression in melanoma. Broken lines indicate that not all MITF-M targets may be coactivated by indicated epigenetic coactivators.

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2. Regulation of MITF expresion

MITF gene locus encodes a transcription factor of the basic-helix-loop-helix-leucine zipper (bHLH-LZ) type, belonging to a large family of bHLH factors comprising also for example the myc oncogenes. Together with TFE3, TFEB, and TFEC, MITF-M constitutes a small bHLH subfamily sharing high sequence similarity. MITF gene was identified twenty years ago and cloned from a microphthalmic and hypopigmented mutant mouse having transgene-insertion at the MITF locus (Hodgkinson et al., 1993). The MITF locus has at least nine promoters producing corresponding MITF isoforms which differ in the first exon and share exons 2-9 (reviewed by Steingrimsson et al., 2004; Levy et al., 2006). Only the MITF-M isoform is melanocyte-specific and expressed exclusively in melanocytes and melanoma cells, whereas some other tissues (osteoclasts, mast cells, heart muscle) express other isoforms of MITF. MITF-M determines the specification of the melanocyte lineage during the embryonic development and stands centrally in the transcriptional network of pigment cells, regulating a number of genes involved not only in melanocyte differentiation and pigment formation (Vachtenheim and Borovansky, 2010; Yajima et al., 2011), but also in the survival, migration, proliferation, invasion and metastasis of melanoma cells (Figure 1).

Regulation of MITF-M transcription is complex and several positive regulators activate the expression (Figure 1), having the corresponding binding motifs in the melanocyte-specific MITF-M promoter. Transcription factor LEF-1/TCF, one of the effectors of the β-catenin pathway, activates MITF-M promoter (Yasumoto et al., 2002). This pathway has been demonstrated to be highly activated in melanoma cells with MITF-M being the necessary mediator of the final pro-survival cellular effects (Widlund et al., 2002; Sinnberg et al., 2011). Transcription factors Sox10 and Pax3 also upregulate MITF-M transcription (Watanabe et al., 1998; Potterf et al., 2000). Sox10 is also stimulated by protein kinase TYRO3, another positive MITF-M regulator in melanoma (Zhu et al., 2009).

CREB is an another transcription factor activating MITF-M expression. The αMSH hormone increases cAMP level, therefore activates PKA (protein kinase A) and the consequence is an increased MITF-M expression and pigmentation. Recently, it has been described that TGF-β represses protein kinase A activity and therefore reduces the CREB-dependent transcription of the MITF-M promoter (Pierrat et al., 2012). CREB requires the coactivators p300/CBP. These proteins, which are histone acetyltransferases, interact with MITF-M and are believed to coactivate at least some MITF-M target genes (Sato et al., 1997; Price et al., 1998). Furthermore, the phosphorylation of serine 73 is required for MITF-M-p300 interaction, at least after the Kit signaling (Hemesath et al., 1998). Controversially, however, it has been observed that MITF-M with mutation of this serine and the N-terminus truncated (including the S73) MITF-M construct were fully capable of activating promoter-reporter and the endogenous target tyrosinase (Vachtenheim et al., 2007).

Whereas the role of p300/CBP in coactivating endogenous targets might be disputable, another prominent epigenetic mechanism has been found to be crucial for MITF-M expression. The chromatin remodeling complex SWI/SNF has been found to be essential for the MITF-M expression in melanoma cells (Vachtenheim et al., 2010). The chromatin remodeling complex

SWI/SNF is composed of about 10-12 proteins and exists in the cells as several subcomplexes capable of changing the local structure of chromatin at the promoter sites. It has important functions in expression of various lineage specific genes and plays an essential function in basic cell processes that include mainly regulation of transcription, DNA replication and repair, and homologous recombination. The SWI/SNF is strongly implicated in human cancer, as several SWI/SNF subunits has been reported missing or strongly downregulated in tumors, and numerous experimental findings suggest that this complex functions as a tumor suppressor (reviewed by Reisman et al., 2009; Wilson and Roberts, 2011). The complex requires at least one ATPase subunit (Brg1 or Brm) and the presence of ATPase Brg1 and Brm is mutually exclusive in the subcomplexes. The components of SWI/SNF are generally highly expressed in melanoma cell lines and at least one ATPase of the complex, Brg1 or Brm, is always present in melanoma cell lines (Vachtenheim et al., 2010; Keenen et al., 2010). The positive effect of SWI/SNF on MITF-M transcription is direct, since both Brg1 and Brm were recruited to the MITF-M promoter in vivo, as revealed by chromatin immunoprecipitation assays (Vachtenheim et al., 2010). In addition, it is possible that SWI/SNF augment the MITF-M expression through LEF-1/TCF because Brg1 interacted with β-catenin and the SWI/SNF complex enhanced transcription of LEF-1/TCF downstream genes (Barker et al., 2001). Recently, we have found (Ondrušová et al., submitted) that Brg1 knockdown in melanoma cells downregulates also Sox10, an upstream MITF-M regulator, so this mechanism might also contribute to the MITF-M regulation. It is intriguing that for most cancers, SWI/SNF complex is a clear tumor suppressor, whereas a small number of cancer types has been recognized (prostate cancer, gastric and colorectal cancer, and melanoma) for which the SWI/SNF seems to function as a tumor promoter, probably by upregulating genes required for proliferation, antiapoptotic activity, invasivity or other characteristics of cancer cells.

It should be noted that the cut-homeodomain transcription factor Onecut-2 (OC-2) also stimulates MITF-M promoter activity. It binds to its promoter (although no OC-2 binding sites were found in the promoter region) and overexpression of ectopic OC-2 in transfected cells stimulates MITF-M promoter activity. Moreover, OC-2 is expressed in melanocytes (Jacquemin et al., 2001). We observed that even cdk inhibitor p21, which itself is a target of MITF-M, is its transcriptional activator (Sestakova et al., 2010). It probably stimulates the CREB pathway and is widely expressed in melanoma cell lines and tumors, and presumably protects malignant cells against apoptotic stimuli.

The POU domain containing transcription factor Brn-2 (N-Oct-3) has been recognized as a repressor of MITF-M expression in melanoma cells (Goodall et al., 2008). Brn-2 bound to MITF-M promoter both in gel-shift assays and in chromatin immunoprecipitation, indicating a direct repression. An inverse correlation between Brn-2 and MITF-M expression was found in melanomas and transfection assays showed that expression of exogenous Brn-2 substantially repressed the MITF-M promoter-reporter. Brn-2 also marked the subpopulation of low MITF-M cells in melanomas (which are heterogenous in MITF-M expression) and these cells displayed low proliferation but high invasiveness (also see below).

One of the effectors of the Hedgehog signaling, transcription factor GLI2, which also mediates the effect of the TGF-β/SMAD pathway, is another repressor of MITF-M expression (Javelaud

http://dx.doi.org/10.5772/55191

et al., 2011). GLI2 expression inversely correlates with MITF-M expression in melanoma cell lines, and GLI2 and M-MITF represses each other's expression (Javelaud et al., 2011). The GLI2 expressing cells with low MITF-M level resemble in biological properties the Brn-2 positive tumor cells (see above), having more invasive and migratory behavior. The Hedgehog pathway is derailed in melanoma (Stecca et al., 2007) acting mainly via GLI1 but does not have inhibitory activity on MITF-M expression. Obviously more investigation is required to elucidate the function of the Hedgehog and TGF-β/SMAD pathways and GLI effectors in melanoma.

3. Downstream targets of MITF

A large number of genes is transcriptionally regulated by MITF-M. Which genes are activated also depends on how high is the level of MITF-M and whether the regulation occurs in normal or malignant melanocytes (Goodall et al., 2008; Carreira et al., 2006; Javelaud et al., 2011). Intriguingly, besides many pro-proliferative and pro-survival genes that are regulated by MITF-M, cell cycle inhibitors p21 and p16 are also its targets (Carreira et al., 2005; Loercher et al., 2005), presumably mainly in normal melanocytes or at higher levels of MITF-M, which favor differentiation (Figure 1). MITF-M interacts with the Rb protein (Yavuzer et al., 1995) which cooperates with MITF-M to induce p21 (Carreira et al., 2005). Importantly, MITF-M regulates almost all genes the products of which are involved in specific melanocyte differentiation, i.e. the formation of the pigment melanin and its deposition into melanosomes. The enzymes involved in melanin formation are tyrosinase and tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2, also known as dopachrome tautomerase, Dct). Even many genes encoding structural melanosomal proteins and factors involved in the melanosome motility are regulated by MITF-M (reviewed in Vachtenheim and Borovansky, 2010). Interestingly, exceptional melanoma cell lines which are transcriptionally silent for MITF-M and do not express the downstream genes tyrosinase, TRP-1, and TRP-2, do not express these markers even after the exogenous transfer of MITF-M, so these cell lines are MITF-M nonresponsive (Vachtenheim et al., 2001). It is therefore likely that the loss of all melanoma markers did occur in these cell lines concomitantly with their absolute dedifferentiation during the loss of MITF-M expression, after which they in fact do not biochemically resemble melanomas.

MITF-M transcriptionally activates a number of genes that act prosurvivally in melanomas. The cyclin dependent kinase 2, which proved crucial in melanoma proliferation (Du et al., 2004) is an important target of MITF-M. MITF-M further regulates HIF1α, a hypoxia response factor (Busca et al., 2005) and APE-1/Ref-1, through which MITF regulates cellular response to ROS (Liu et al., 2009). The c-Met, a receptor kinase for the hepatocyte growth factor, has proinvasive features and promotes metastatic process in several tumors including melanoma. c-Met has been shown to be a target of MITF-M. Gel shift assays with melanoma cell nuclear extracts demonstrated binding by endogenous MITF-M protein to the c-Met promoter at the consensus MITF-M binding site and exogenous MITF-M activated the c-Met expression (McGill et al., 2006). Additionally, MITF-M is a transcriptional activator of Tbx2 gene (Carreira et al., 2000). T-box family of transcription factors are crucial in embryonic development. Tbx2

has been implicated in morphogenesis of a wide range of organs including limbs, kidneys, lung, mammary gland, and heart. Tbx2 is expressed in melanoma cell lines in positive correlation with MITF-M. Tbx2 promoter contains a consensus MITF-M recognition element to which MITF-M is recruited and activates Tbx2 expression (Carreira et al., 2000). As Tbx2 is overexpressed in several cancers including melanoma and plays a role in suppressing senescence, MITF-M modulates the survival of melanoma cells also via this factor.

MITF-M also upregulates the expression of clearly antiapoptotic genes. First, it activates the general antiapoptotic protein Bcl-2 (McGill et al., 2002), which is important for the survival of both melanoma and melanocyte lineage. Second, melanoma inhibitor of apoptosis (ML-IAP, livin) is a potent inhibitor of apoptosis in melanoma and is directly regulated by MITF-M (Dynek et al., 2008). It was shown that MITF-M was recruited to the ML-IAP promoter by chromatin immunoprecipitation and gel shift assays, activated ML-IAP promoter-reporter, and downregulation of MITF-M abrogated ML-IAP expression. The SLUG protein was reported to be a crucial determinant of melanoma metastasis, and its gene is also upregulated by MITF-M (Gupta et al., 2005). Another important gene regulated by MITF-M is Dia (DIAPH1, encoding the diaphanous-related formin Dia1). Increased MITF-M level favors expression of Dia and higher proliferation of melanoma cells. On the contrary, lower MITF-M downregulates Dia but the cdk inhibitor p27 is stabilized resulting in slower proliferation but higher invasive potential. Thus, a "rheostat" model is proposed where MITF-M levels determine the proliferation, differentiation, and invasiveness of melanoma cells (Carreira et al., 2006). Noteworthy, many other genes were identified as potential MITF-M targets, by the microarray analysis (Hoek et al., 2008). The SWI/SNF complex has been shown to be an important coactivator for many target genes, especially those involved in pigment formation, in human melanoma cell lines (Keenen et al., 2010). Of the two ATPases of the complex, Brg1 seems to be more important than Brm since its knockdown downregulates the MITF-M target genes more efficiently than Brm knockdown (Vachtenheim et al., 2010).

MITF-M is considered to be a melanoma oncogene because mutations and amplifications were found in patients´ samples (Garraway and Sellers, 2006; Levy et al., 2006; Cronin et al., 2009). Copy gains at the MITF locus were found in about 10% melanoma samples and mutations of the MITF-M pathway were also described, even in the Sox10 gene (Cronin et al., 2009). Recently, novel MITF-M mutation, E318K, has been identified. This mutation is a germline allele variant (Yokoyama et al., 2011). E318K MITF-M encodes a protein with impaired sumoylation of MITF-M and differential regulation of several MITF-M targets. For example, the mutant enhances MITF-M protein binding to the HIF1α promoter, a MITF-M known target, and increases its transcriptional activity (Bertolotto et al., 2011; Yokoyama et al., 2011). The presence of this allele constitutes more than fivefold risk factor for melanoma and renal cell carcinoma. Further, the mutant protein enhanced melanocytic and renal cell carcinoma clonogenicity, migration and invasion, further implicating this MITF-M variant as a risk factor for these two cancers (Bertolotto et al., 2011). Together, as MITF-M is strictly required for the embryonic development of melanocytes, maintains the survival of melanoma, and gene amplifications and mutations in the protein coding region have been found, it is considered as a lineage addiction oncogene.

http://dx.doi.org/10.5772/55191

Acknowledgements

This work was supported by a grant NT/11229-3 (to J.V.) from the Ministry of Health (IGA, MH) and the institutional research project PRVOUK-P25/LF1/2. L.O. participates in the PhD program at Charles University in Prague, Third Faculty of Medicine, Czech Rep.

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Conflict of interest The authors state no conflict of interest.

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Functional interaction of histone acetyltransferases PCAF and hGCN5 with microphthalmia-associated transcription factor in melanoma cells

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Keywords:

PCAF, hGCN5, melanoma, MITF

Footnotes:

The study was supported by grant NT11229-3/2010 from the Ministry of Health of the Czech Republic

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Abstract

Melanoma cell lineages express microphthalmia-associated transcription factor (MITF), a transcriptional activator containing the basic-helix-loop-helix (bHLH) domain. The melanocyte-specific isoform of MITF is critical for differentiation, proliferation, and survival of both normal melanocytes and melanoma cells. Numerous genes have been identified as targets of MITF in malignant melanocytes. We demonstrate that histone acetyltransferases (HATs) PCAF (p-300/CBP-associated factor) and hGCN5 (human general control nonrepresible-related 5) interact with MITF *in vitro* and in cells, and that the transactivation domain and bHLH region of MITF are essential for this interaction. The binding is direct and does not involve complex formation with p300 coactivator, a known MITF-interacting protein. Overexpression of PCAF or hGCN5 resulted in the inhibition of activity of several MITF-responsive promoter-reporters, and siRNA directed to PCAF activated the MITF target melastatin promoter in 501mel melanoma cells. This indicates that the endogenous level of PCAF is inhibitory for MITF transcriptional activity. Further, the HAT-defective mutants of PCAF and hGCN5, which still effectively bound to MITF, inhibited the target promoter-reporters as strongly as the wild types, indicating that acetyltransferase activity is not necessary for the inhibition. These results suggest that the two HATs, PCAF and hGCN5, may modulate MITF activity independently of their acetyltransferase function.

Introduction

Malignant melanoma is a highly invasive and early metastasizing tumor and is known for its resistance to conventional anticancer therapies (Gray-Schopfer et al., 2007). MITF (microphthalmia-associated transcription factor) is a pivotal transcriptional regulator of normal and malignant melanocytes, which drives the basic processes of melanoma biology (Vachtenheim and Borovansky). MITF has been shown to be amplified in 10-20 % of melanoma metastases (King et al., 2001) and to potentially act as a melanoma oncogene (Garraway et al., 2005).

MITF has many isoforms differing in the first exon, but downstream exons are common to all isoforms. Each of these isoforms (-A, -B, -E, -N, and –H) utilizes its own tissue-specific promotor for transcription (Shibahara et al., 2001). The M-isoform is expressed exclusively in the melanocyte lineage and here is referred to as MITF.

Histone acetyltransferase enzymes P300/CBP interact with MITF *in vitro* and in cells and are believed to activate transcription of the MITF targets, possibly by acetylating the histones in nucleosomes within the target promoters (Sato et al., 1997; Price et al., 1998). Two transcription activation domains (TADs) have been identified in MITF: a strong N-terminal TAD (amino acids 114-135) (Sato S et al., 1997) and a C-terminal TAD (amino acids 324-369) (Takeda K et al., 2000). The CBP was shown to interact with the N-terminal TAD of MITF (Sato S et al., 1997).

Here we demonstrate that other HATs PCAF (p-300/CBP-associated factor) and hGCN5 (human general control nonrepresible-related 5) interact with MITF *in vitro* and in cells and inhibit MITF activity independently of their acetyltransferase function.

Results

MITF specifically interacts with PCAF and hGCN5 *in vitro* and *in vivo*

MITF is a b-HLH-LZ type transcription factor and is related to other transcription factors of the Mi-family, TFE3, TFEC and TFEB (Hemesath et al., 1994; Rehli et al., 1999). MITF possesses two transcription activation domains (TADs), but the TAD located at the N-terminus is much stronger than the C-terminal TAD. Pull-down assays using the *in vitro* translated MITF mutants (generated as described in Fig. 1A) showed that MITF specifically interacts with PCAF and hGCN5 *in vitro* and the bHLH region is strictly required for this association. We have found similar binding profiles for PCAF and hGCN5 and compared the binding with the binding of MITF to retinoblastoma protein (Rb), which was shown to interact with MITF (Carreira et al., 2005). Interestingly, as shown in Fig. 1B, the MITF N-terminal TAD is required for binding together with bHLH (compare mutants 106-294 and 144-292) only if the most of the N-terminus is absent, while it is dispensable if the N-terminus is present (mutant del 106-170). The N-terminus has weaker autonomous binding activity (mutant 1-116), which might explain the binding potency of MITF mutant del 106-170. The binding of MITF to GST-PCAF and GST-hGCN5 *in vitro* was confirmed by pull down utilizing the nuclear extracts from 501mel melanoma cells (Fig. 1C). Immunoprecipitation revealed that both PCAF and hGCN5 interact with MITF *in vivo* (Fig 1.D). Thus, together these results suggest that both PCAF and hGCN5 can form a complex with MITF in melanoma cells, and the most important region for this interaction seems to be the bHLH region.

Overexpression of PCAF or hGCN5 leads to repression of the activity of MITF-responsive melastatin promoter.

We wished to investigate whether the MITF-responsive melastatin promoter is activated by PCAF, hGCN5 or their HAT null-mutants. Surprisingly, overexpression of PCAF or hGCN5 repressed the MITF-responsive melastatin reporter in 293T cells (Fig. 2). The histone acetyltransferase activity of PCAF and hGCN5 was not required for the repression as HAT-null mutants of both enzymes repressed as strongly as their wild types. As shown in Fig. 3, the activity of melastatin promoter was downregulated by PCAF, hGCN5 and their HAT null-mutants in a decreasing manner with their increasing amount. Thus, taken together, wild-type enzymes and HAT-null mutants are effective repressors.

TFE3 transcription factor reacts to PCAF and hGCN5 differently to regulate melastatin promoter

Next, we explored whether PCAF and hGCN5 affect the melastatin promoter activated by TFE3, which is closely related to MITF. Expression vectors for TFE3 and PCAF or hGCN5 were co-transfected with melastatin promoter-reporter into 293T cells. As shown in Fig. 4, co-transfection of hGCN5 (or HAT-null hGCN5 mutant) decreased the melastatin promoter activity. Only slight inhibition was seen with PCAF, whereas the HAT-dead mutant of PCAF revealed no inhibitory activity on the promoter. We have also found that other MITF-responsive promoters such as tyrosinase and TRP1 are differently regulated by PCAF and hGCN5 (data not shown). Therefore, PCAF and hGCN5 might act as selective coregulators of promoters activated by the MITF family members.

Endogenous level of PCAF inhibits melastatin promoter

As overexpression of PCAF was effective in repression of the melastatin promotor-reporter directed by exogenous MITF in 293T cells, we decided to deplete endogenous PCAF in melanoma cells. The siRNA duplex that targets PCAF was used to knockdown PCAF expression in 501mel melanoma cells and was seen to activate the melastatin promoter (Fig. 5). This suggests that the endogenous PCAF protein is an inhibitor of transcription mediated by MITF in melanoma cells.

Discussion

PCAF and hGCN5 exhibit histone acetyltransferase activity and play vital roles in transcription. In addition to global and gene-specific HAT activities, PCAF and hGCN5 have been shown to acetylate numerous transcription factors directly (Nagy and Tora, 2007). The results presented here demonstrate an important role for PCAF and hGCN5 in MITF-directed transcription. Both of them directly bind to MITF. The bHLH and transactivation domains of MITF are required for these interactions, indicated by pull-down analysis. Further, PCAF and hGCN5 function as transcriptional repressors of MITF in a HAT activity-independent manner. However, PCAF and hGCN5 are mostly presented as transcriptional activators. Most of their effects are dependent on their acetylation activity and can lead to protein stabilization, such as in the case of c-MYC (Patel et al., 2004) or p53 (Liu et al., 1999). On the other hand, their HAT-dependent repressive activity has also been described. We showed that both, wild-type versions and HAT-deficient mutants of PCAF and hGCN5 were able to repress the MITF-responsive melastatin promoter. Because HAT activity is not necessary for this inhibition, they probably modulate the transcriptional activity of MITF by physical interaction. Nevertheless, we have found previously that PCAF acetylates MITF (data not shown). Similar results were described by Yoshimochi (2010) in the case of transcription factor FOXO. We also explored the activity of melastatin promoter directed by endogenous MITF after overexpression of PCAF and hGCN5 in melanoma cells and no inhibition was seen (data knot shown). However, the activity of melastatin promoter was increased after PCAF knockdown by the siRNA duplex directed to PCAF. Thus, the endogenous level of PCAF is inhibitory. It seems that a higher dose of PCAF than the endogenous level is irrelevant for melanoma cells. The distinct effect of PCAF and hGCN5 on the activity of melastatin promoter directed by TFE3, MITF closely related transcription factor, is not so surprising because PCAF and hGCN5 are subunits of multiprotein complexes with different substrate specificity (Nagy and Tora, 2007). In conclusion, we identified PCAF and hGCN5 as novel repressors of MITF. The suppressive function is independent of their HAT activity. Further study is required for elucidating the mechanism of the transcriptional inhibition.

Materials and methods

Plasmid constructs and shRNA

MITF wild-type cDNA and its mutants were cloned in the pCMV vector. pCMV-MITF plasmid was created by in frame cloning of the MITF open reading frame sequence. Deletions were introduced by PCR mutagenesis with the Pfu DNA Turbo polymerase (Stratagene). The plasmid pCI.PCAF was kindly supplied by Dr. V. Ogryzko. The expression plasmid hGCN5 was obtained from Origene. Mutants PCAFΔHAT and hGCN5(E575Q) were generated by Quick-change mutagenesis kit (Stratagene). Both HAT mutants were verified to have no or very low activity by the HAT assay using ^{3}H-acetyl-CoA. Amino-terminal FLAG-tagged wt PCAF, wt hGCN5, and their HAT mutants were cloned in pFLAG-CMV-6c (Sigma Aldrich). The human melastatin promoter spanning sequences from –1458 to +161 (relative to transcription start) was cloned in pGL3-basic (Promega). All plasmid constructs were verified by DNA sequencing of both strands.

The siRNA duplex directed to PCAF and the non-targeting siRNA duplex used as a negative control were purchased from Thermo Scientific Molecular Biology.

Cells and transfections

501mel cells were a gift from Dr. R. Halaban and were maintained in RPMI 1640 medium with 10% fetal bovine serum, antibiotics, and L-glutamine. The 293T cells (Genecopoeia) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, antibiotics, and L-glutamine. 293T cells were transfected by the calcium phosphate method in 12-well plates and 501mel cells were transfected using Lipofectamine 2000 (Invitrogen). To normalize luciferase activities, pCMV-ß co-transfection followed by estimation of ß–galactosidase activity or dual luciferase assay system (Promega) were used.

GST-fusion proteins and pull-down assays

The GST-fusion constructs for bacterial expression of GST-PCAF, GST-hGCN5, GST-Rb or empty GST as a control were created by cloning sequences into pGEX-4T1 plasmid (Amersham) and expressed in *E.coli* strain BL-21Star(DE3)-LysS (InVitrogen) as described (Vachtenheim at al., 2007). Pull-down reactions were carried out with either the nuclear extracts from 501mel cells or the proteins synthesized *in vitro* by using TNT reticulocyte lysate-based coupled IVT system (Promega) with ^{35}S-methionin (Amersham Biosciences). Preadsorbed cell extracts or ^{35}S-labeled proteins were mixed with the GST-fusion protein-

Sepharose beads and after incubation and washing were resolved by SDS-PAGE. Autoradiography or immunoblotting were used for the detection. Anti-MITF (D5) (Labvision) was used to probe Western blots.

Immunoprecipitation

For immunoprecipitations, cells were lysed in Nonidet P-40 lysis buffer (50 mM TRIS pH 7.5, 150 mM NaCl, 1.0 % Nonidet P-40) containing protease inhibitors, and 300 µg of protein was immunoprecipitated with anti-PCAF (Abcam), anti-hGCN5 (Santa Cruz Biotechnology), anti-LEF1 (Millipore) antibody or rabbit or mouse IgG (Sigma) overnight at 4 °C. Following overnight incubation complexes were affinity purified by incubation with 50 µl of Protein-G or Protein-A beads (Santa Cruz Biotechnology) for 1 hour at 4 °C, washed five times in lysis buffer and size fractionated using 10% SDS-PAGE. MITF was detected by Western blot analysis using anti-MITF antibody (D5).

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Figure legends

Fig. 1 MITF specifically interacts with PCAF and hGCN5 *in vitro* and *in vivo*

A. Schematic diagram of the full-length MITF and deletion constructs. TADs are transcription activation domains. M is a short N-terminal melanocyte-specific sequence.
B. Pull-down experiments demonstrating interaction of PCAF, hGCN5 and Rb with the MITF deletion mutants *in vitro*. Pull-down assays were carried out with the *in vitro* translated MITF mutants (generated as shown in Fig. 1A) and GST-hGCN5, GST-PCAF, and GST-Rb fusion proteins expressed and purified from bacteria and bound to glutathione-sepharose beads.
C. Pull-down experiments demonstrating the interaction of PCAF and hGCN5 with MITF *in vitro* utilizing the nuclear extracts from 501mel melanoma cells. For MITF detection, the protein bound to the beads was separated by SDS-PAGE and immunoblotted with antibody anti-MITF (D5).
D. Both, PCAF and hGNC5 interact with MITF *in vivo*. Extracts from 501mel cells were immunoprecipitated with the anti-MITF antibody (D5), control IgG, irrelevant antibody, or anti-PCAF (or anti-hGCN5) antibody as a positive control, and detected with antibodies to PCAF or hGCN5.

Fig. 2 Overexpression of PCAF, hGCN5, or their HAT-null mutatns leads to repression of the activity of MITF-responsive melastatin promoter.

Empty vector (lanes 1,4,7,10) or FLAG-tagged versions of PCAF and hGCN5 (lanes 2,5,8,11) or their HAT-deficient mutants (lanes 3,6,9,12) were co-transfected with the melastatin promoter-luciferase construct into 293T cells. The expression of PCAF and hGCN5 proteins was comparable (W. blot). To verify that HAT-null mutants do not possess HAT activity, the *in vitro* HAT assay was performed with the GST-fusion proteins and histones as substrates (lower part of the figure).

Fig. 3. Transcriptional repression of the melastatin promoter by PCAF and hGCN5 is dose-dependent.

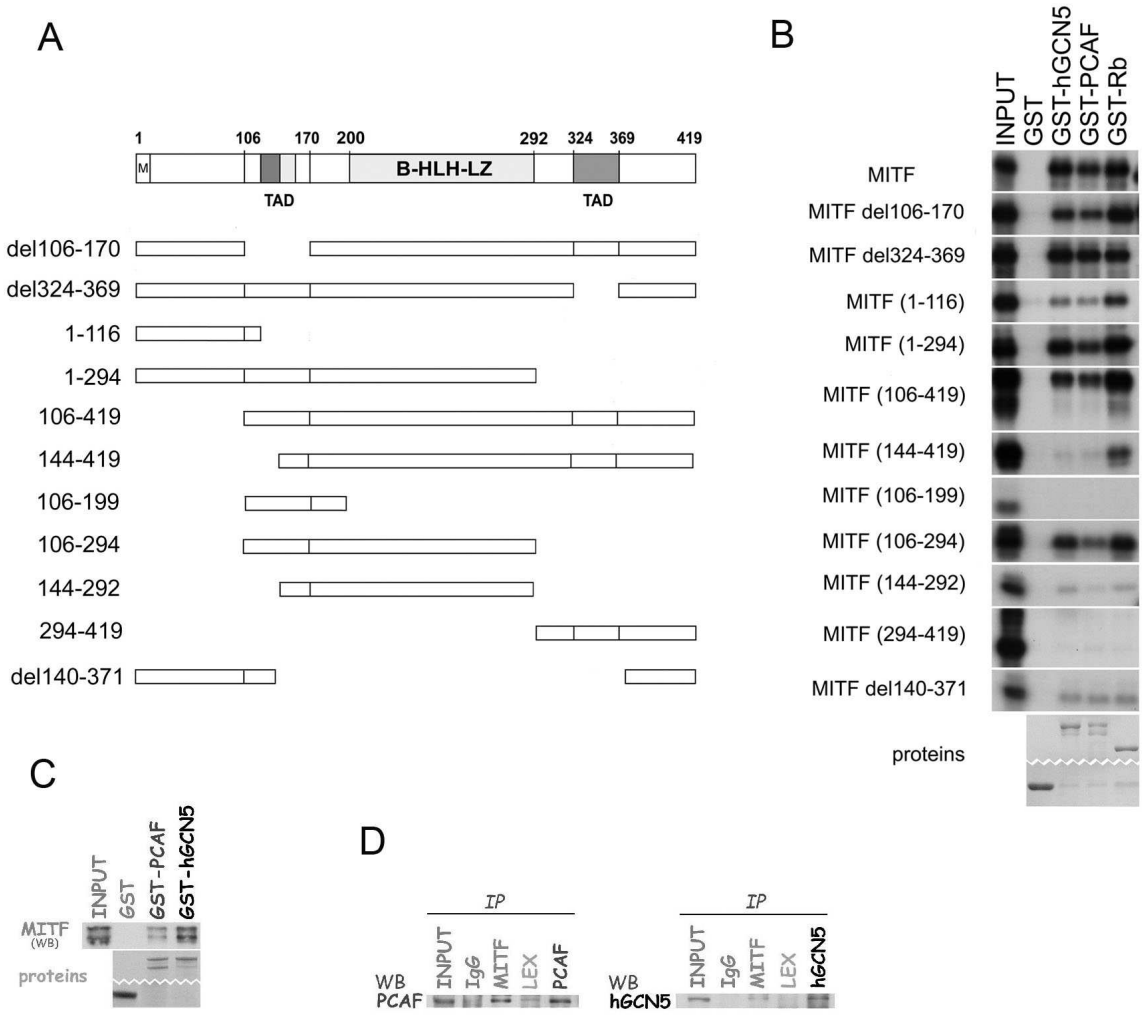
293T cells were transfected with increasing amounts of the indicated plasmid and melastatin promoter-luciferase construct.

Fig. 4. TFE3 also activates melastatin promoter and its activity is repressed by co-transfected hGCN5 (and HAT-null hGCN5 mutant), but not by PCAF.

TFE3 expression vector was co-transfected with the melastatin promoter-luciferase construct into 293T cells.

Fig. 5. The siRNA duplex that targets PCAF activates MITF-responsive promoter melastatin in 501mel cells.

The melastatin promoter-luciferase construct and the siRNA duplex to MITF or PCAF were transiently co-transfected in 501mel melanoma cells and luciferase activity was determined 2 days later.


A
1
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292
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369
419
M
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TAD
TAD
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del324-369
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106-419
144-419
106-199
106-294
144-292
294-419
del140-371
B
INPUT
GST
GST-hGCN5
GST-PCAF
GST-Rb
MITF
MITF del106-170
MITF del324-369
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MITF (1-294)
MITF (106-419)
MITF (144-419)
MITF (106-199)
MITF (106-294)
MITF (144-292)
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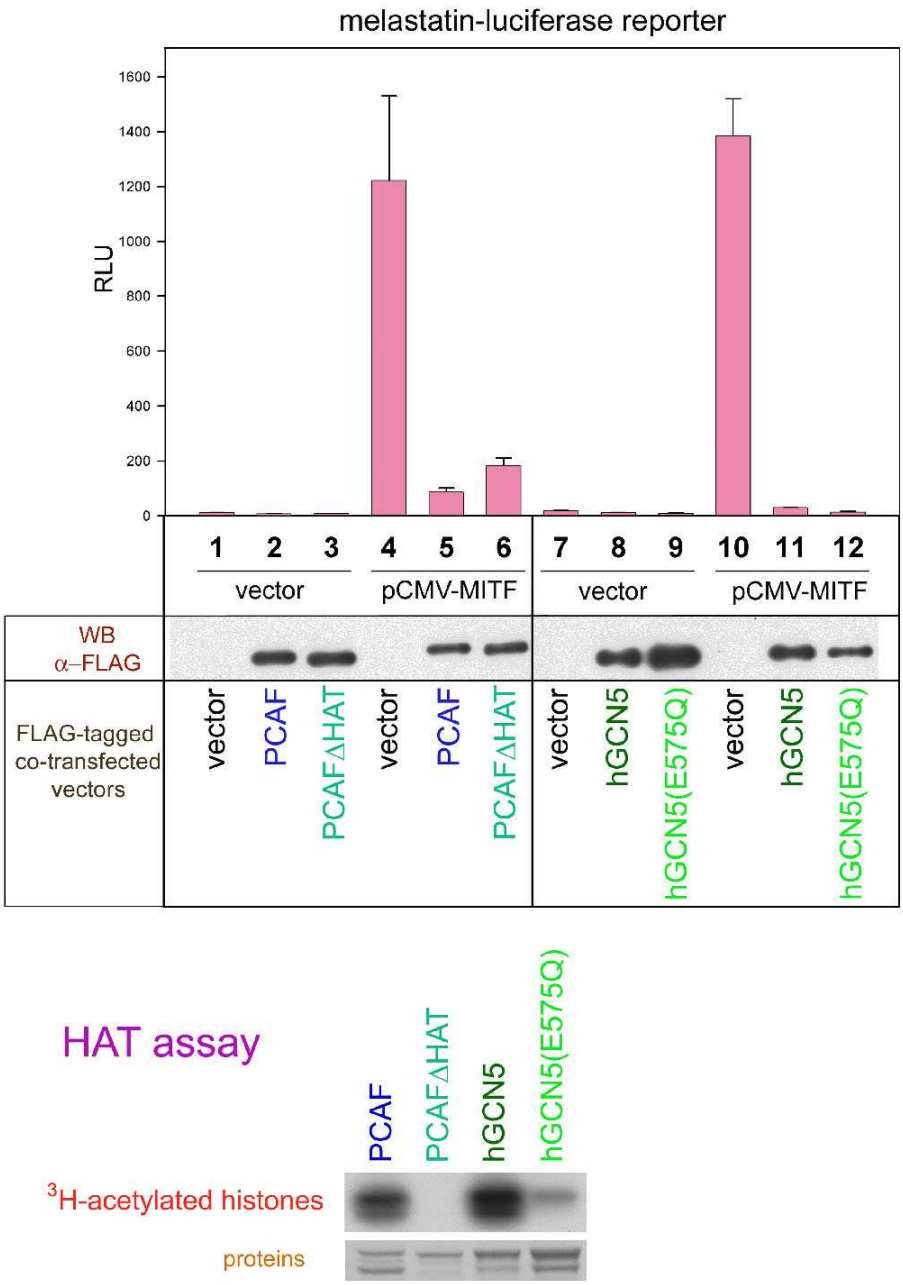
Figure 1


melastatin-luciferase reporter
RLU
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600
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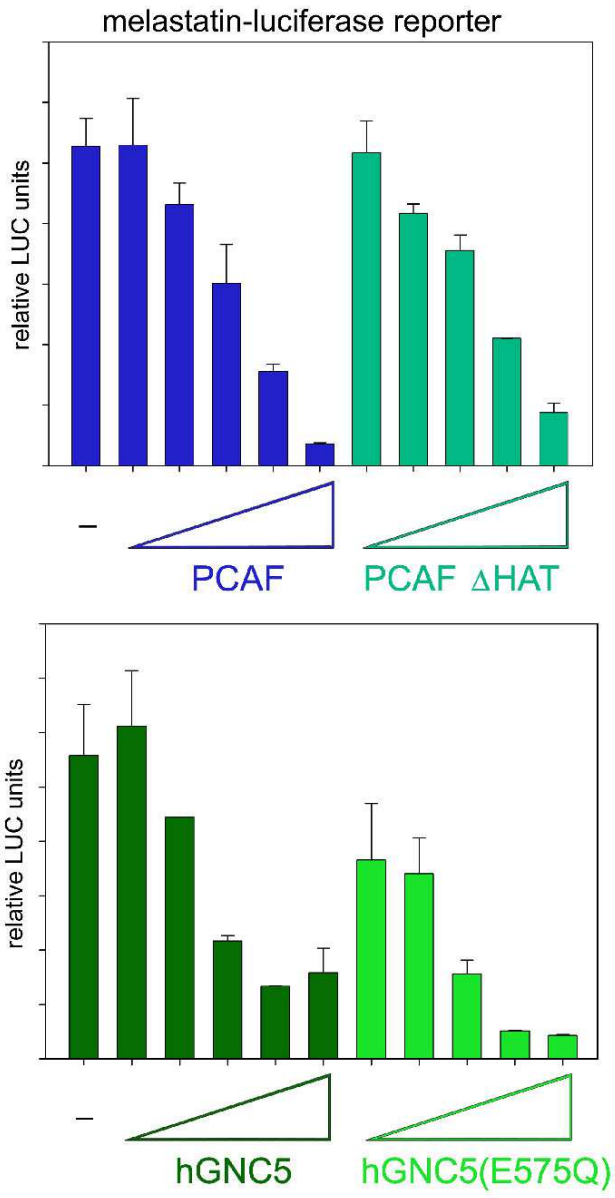
pCMV-MITF
vector
pCMV-MITF
WB
α–FLAG
FLAG-tagged
co-transfected
vectors
vector
PCAF
PCAFΔHAT
vector
PCAF
PCAFΔHAT
vector
hGCN5
hGCN5(E575Q)
vector
hGCN5
hGCN5(E575Q)

HAT assay
PCAF
PCAFΔHAT
hGCN5
hGCN5(E575Q)
3H-acetylated histones
proteins

Figure 2


melastatin-luciferase reporter
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PCAF
PCAF ΔHAT
relative LUC units
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hGNC5
hGNC5(E575Q)

Figure 3

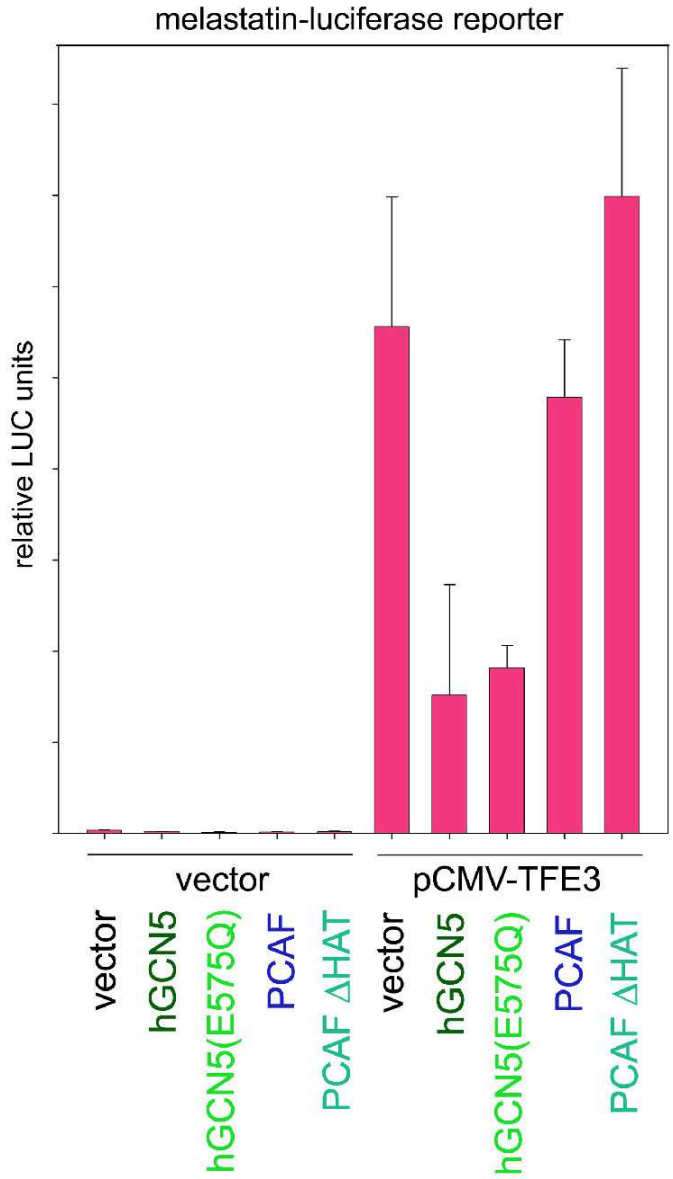
melastatin-luciferase reporter
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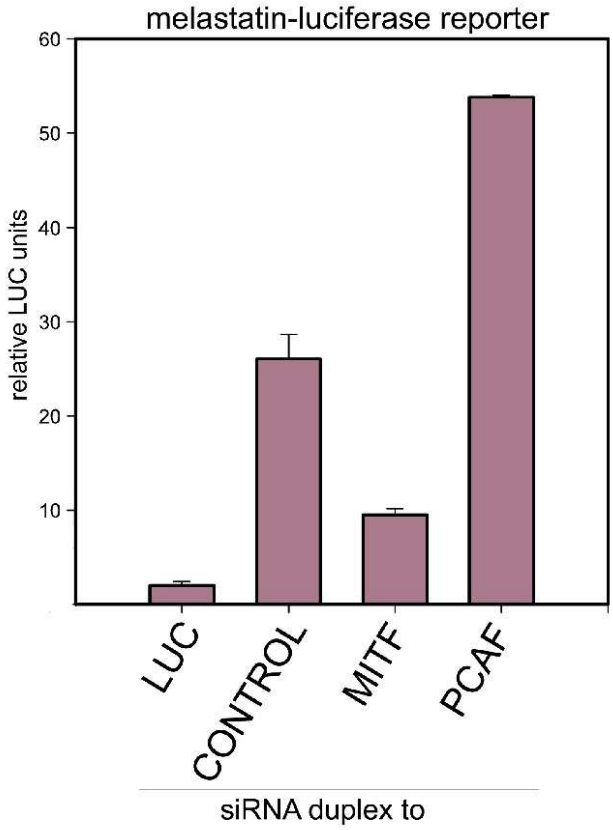
Figure 4


melastatin-luciferase reporter
relative LUC units
60
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PCAF
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Figure 5