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Analýza vlivu mutací v genu pro nukleofosmin na jeho interakční potenciál

The impact of *nucleophosmin* gene mutations on its interaction potential

Dizertační práce

Školitelka: Mgr. Barbora Brodská, Ph.D.

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Prohlášení:

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Mgr. Markéta Šašinková

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ABSTRAKT

Nukleofosmin 1 (NPM1; nucleophosmin 1) se v buňce vyskytuje převážně v jadérku, ve formě oligomerů tvořených skrze N-koncovou doménu (NTD; N-terminal domain), a jako molekulární chaperon a transportní protein má široké spektrum interakčních partnerů včetně p53 či p14Arf. Mutace v C-koncové oblasti NPM1 je prokazována asi u 30 % pacientů s akutní myeloidní leukémií (AML; acute myeloid leukemia) a projevuje se aberantní expresí mutovaného (mut; mutated) NPM1 v cytoplazmě. V důsledku toho dochází v leukemických buňkách s mutNPM1 k přesunu mnoha proteinů s ním interagujících, včetně jeho přirozené formy (wt; wild-type), do cytoplazmy. Pro detekci interakcí a oligomerních komplexů NPM1 jsme zavedli a optimalizovali jak *in vitro* techniky – nativní a seminativní gelovou elektroforézu a imunoprecipitaci, tak testování *in vivo* – konfokální mikroskopii a metody časově rozlišené fluorescence. Těmito metodami jsme prokázali, že v důsledku C-terminální mutace NPM1 dochází k narušení jeho interakce s jadérkovým proteinem nukleolinem (NCL; nucleolin), která se tvoří mimo NTD, a že komplex mutNPM1-NCL nevzniká ani po ošetření buněk cytotoxickými léčivy navozujícími relokalizaci obou partnerů do stejného buněčného kompartmentu. V naší studii dále ukazujeme, že oligomery mutNPM1 jsou méně stabilní než oligomery wtNPM1, což je poznatek důležitý pro vývoj léčiv cílených na oligomerizaci NPM1. Interakční vlastnosti NPM1 totiž úzce souvisejí s jeho oligomerizačním stavem a zásah do NTD NPM1 může zajistit zeslabení interakce mutNPM1 s interakčními partnery a v důsledku toho i obnovení jejich správné lokalizace a funkce. Zkonstruovali jsme proto několik forem NPM1 s pozměněnou či zkrácenou NTD a popsali jsme vliv těchto zásahů na schopnost takto mutovaných molekul oligomerizovat nebo interagovat s wtNPM1. Zjistili jsme, že zatímco bodové mutace oligomerizaci ovlivní jen málo, zkrácení NTD tvorbě komplexů účinně zabraňuje. Všechny mutované formy ale byly do jisté míry schopny koprecipitovat wtNPM1. Nakonec prezentujeme nové poznatky o vlastnostech léčiva NSC348884, které je deklarováno jako inhibitor oligomerizace NPM1. V doporučených koncentracích toto léčivo vyvolávalo u všech leukemických buněčných linií apoptózu, zároveň jsme však prokázali přítomnost neporušených NPM1 oligomerů. Naše experimenty dále odhalily schopnost NSC348884 zabránit přirozené adhezi buněk. Celkově tedy tato práce předkládá způsoby detekce oligomerů NPM1 a analyzuje vliv mutací v N- i C-koncové doméně NPM1 na jeho oligomerizační a interakční potenciál.

Klíčová slova: NPM1, oligomerizace, mutace, AML, NCL, NSC348884, detekce oligomerů

ABSTRACT

Nucleophosmin 1 (NPM1) is predominantly localized in the nucleolus and occurs mainly in oligomers formed through its N-terminal domain (NTD). As a transport facilitator and chaperone, NPM1 has a wide range of interacting partners including tumor suppressors p53 and p14Arf. Characteristic C-terminal mutations in NPM1 are reported in approximately 30 % of acute myeloid leukemia (AML) cases and cause aberrant cytoplasmic localization of mutated (mut) NPM1. As a result, many NPM1-interacting proteins, including wild type (wt) NPM1, are relocalized to the cytoplasm. In order to analyze interactions and the oligomeric state of NPM1, we have introduced and optimized several *in vitro* techniques – native and semi-native polyacrylamide gel electrophoresis and immunoprecipitation – as well as *in vivo* confocal microscopy and time-resolved fluorescence approaches. Using these methods, we revealed that mutations at the C-terminal domain of NPM1 prevent it from binding nucleolar protein nucleolin (NCL), which has previously been shown to interact with the central part of NPM1, and that drug-induced relocation of mutNPM1 to close proximity of NCL does not induce mutNPM1-NCL complex formation. We proved a lowered stability of mutNPM1-formed oligomers as compared to the wtNPM1 ones, which could be useful for NPM1 oligomer-targeting drugs design. NPM1 oligomerization domain also often serves as an interface for interaction with other proteins. Thus, targeting the NPM1 oligomerization domain might weaken mutNPM1 interaction ability and allow the proper localization and function of misplaced proteins to be restored. We therefore constructed NPM1 variants with mutations or deletions within NTD and analyzed their oligomerization characteristics and binding ability to wtNPM1. While point mutations did not cause significant effects on the NPM1 oligomerization, partial or complete deletion of NTD efficiently prevented NPM1 complex formation. Nevertheless, all the N-terminal mutated variants were found to coprecipitate wtNPM1 to some extent. Finally, we examined the effect of NSC348884, a putative inhibitor of NPM1 oligomerization. When administered in recommended concentrations, NSC348884 induced apoptosis of all AML cell lines, but it did not disrupt oligomer formation. Simultaneously we revealed that NSC348884 interfered with adhesion signaling. Overall, this thesis presents methods to analyze the oligomeric state of NPM1 and evaluates the effects of N- and C-terminal mutations on NPM1 oligomerization and interaction.

Key words: NPM1, oligomerization, mutation, AML, NCL, NSC348884, detection of oligomers

OBSAH

SEZNAM ZKRATEK

△100 NmutNPM1		nukleofosmin 1 s delecí prvních 100 aminokyselin
△117 NmutNPM1		nukleofosmin 1 s delecí prvních 117 aminokyselin
△25 NmutNPM1		nukleofosmin 1 s delecí prvních 25 aminokyselin
AA	amino acid	aminokyselina
ActD	Actinomycin D	Aktinomycin D
ALK	anaplastic lymphoma kinase	anaplastická lymfomová kináza
allo-HSCT	allogeneic hematopoietic stem cell transplantation	alogenní transplantace krvetvorných kmenových buněk
AML	acute myeloid leukemia	akutní myeloidní leukémie
AML s mutNPM1		akutní myeloidní leukémie s mutovaným nukleofosminem 1
APE1	apurinic/apyrimidinic endonuclease 1	apurinová/apyrimidinová endonukleáza 1
APL	acute promyelocytic leukemia	akutní promyelocytární leukémie
AR	allelic ratio	alelický poměr
ARA-C	arabinosylcytosine; cytarabine	cytarabin
ATRA	all-trans-retinoic acid	all-trans-retinová kyselina
BCL-2	B-cell lymphoma-2 protein	
CASP-6	caspase-6	kaspáza-6
CASP-8	caspase-8	kaspáza-8
C-MYC	cellular myelocytomatosis oncogene	
ccN&B	cross-correlation number and brightness assay	kros-korelační sledování počtu a jasu fluoreskujících molekul
CK2	casein kinase 2	kaseinová kináza 2
CRM1	chromosomal region maintenance 1	
CTD	C-terminal domain	C-koncová doména

DNMT3α	DNA methyltransferase 3 alpha	DNA metyltransferáza 3 alfa
DOT1L	disruptor of telomeric silencing 1-like	
DOX	doxorubicin	doxorubicin
E2F-1	E2F transcription factor 1	
EFS	event-free survival	přežívání bez sledované události
eGFP	enhanced green fluorescent protein	zesílený zelený fluorescenční protein
eGFP_mutNPM1		mutovaná forma nukleofosminu 1 značená zesíleným zeleným fluorescenčním proteinem
eGFP_wtNPM1		přirozená forma nukleofosminu 1 značená zesíleným zeleným fluorescenčním proteinem
ERK	extracellular signal-regulated kinase	kináza regulovaná extracelulárním signálem
FBL	fibrillarin	fibrilarin
Fbw7γ	F-box/WD repeat domain-containing protein	
FLIM	fluorescence lifetime imaging microscopy	časově rozlišená fluorescenční mikroskopie
FLT3	FMS-like tyrosine kinase-3	
FRET	fluorescence resonance energy transfer	fluorescenční rezonanční přenos energie
GADD45α	growth arrest and DNA damage-inducible gene 45 alpha	
GC	granular component	granulární složka
HDM2	human homolog of mouse double minute 2 protein	
HIF-1	hypoxia-inducible factor 1	hypoxií indukovaný transkripční faktor 1
HIV	human immunodeficiency virus	virus lidské imunodeficience
HLA	human leukocyte antigens	lidské leukocytární antigeny
CHIKV	Chikungunya virus	virus Chikungunya

IDH	isocitrate dehydrogenase	isocitrát dehydrogenáza
IP	immunoprecipitation	imunoprecipitace
ITD	internal tandem duplication	interní tandemová duplikace
LmB	Leptomycin B	Leptomycin B
LSC	leukemia stem cells	leukemické kmenové buňky
MDM2	mouse double minute 2 protein	
MDS	myelodysplastic syndrome	myelodysplastický syndrom
MEF	myeloid Elf-1-like factor	
MEN	menin	menin
MLL	mixed lineage leukemia	
MNs	myeloid neoplasms	myeloidní neoplázie
MRD	minimal residual disease	minimální zbytková choroba
mRFP1	monomeric red fluorescent protein 1	monomerní červený fluorescenční protein 1
mRFP1_mutNPM1		mutovaná forma nukleofosminu 1 značená monomerním červeným fluorescenčním proteinem 1
mRFP1_wtNPM1		přirozená forma nukleofosminu 1 značená monomerním červeným fluorescenčním proteinem 1
mRNA	messenger RNA	mediátorová RNA
mut	mutated	mutovaný
MW	molecular weight	molekulová hmotnost
N6L		NucAnt 6L
NA	nucleic acid	nukleová kyselina
nat PAGE	native polyacrylamide gel electrophoresis	nativní polyakrylamidová gelová elektroforéza
NCL	nucleolin	nukleolin
NES	nuclear export signal	jaderný exportní signál
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells	
NK	normal karyotype	normální karyotyp

NLS	nuclear localization signal	jaderný lokalizační signál
NmutNPM1		nukleofosmin 1 s delecí v N-terminální doméně
NoLS	nucleolar localization signal	jadérkový lokalizační signál
NPM1	nucleophosmin 1	nukleofosmin 1
NRAS	neuroblastoma RAS viral oncogene homolog	
NTD	N-terminal domain	N-koncová doména
OS	overall survival	celkové přežití
PAGE	polyacrylamide gel electrophoresis	polyakrylamidová gelová elektroforéza
PAK1	p21-activated kinase 1	p21-aktivovaná kináza 1
PD-1	programmed cell death protein 1	protein programované buněčné smrti 1
PD-L1	programmed cell death ligand 1	ligand proteinu programované buněčné smrti 1
PIE	pulsed interleaved excitation	pulzně prokládané buzení
PML	promyelocytic leukemia protein	promyelocytický leukemický protein
PTM	post-translational modifications	posttranslační modifikace
PTPN11	protein tyrosine phosphatase non-receptor type 11	
RARα	retinoic acid receptor alpha	receptor kyseliny retinové alfa
rDNA	ribosomal DNA	ribozomální DNA
RFS	relapse-free survival	přežívání bez relapsu
RNAP	RNA polymerase	RNA polymeráza
ROS	reactive oxygen species	reaktivní kyslíkové radikály
rRNA	ribosomal RNA	ribozomální RNA
SDS	sodium dodecyl sulfate	dodecylsíran sodný
semi-nat PAGE	semi-native polyacrylamide gel electrophoresis	seminativní polyakrylamidová gelová elektroforéza
SF3B1	splicing factor 3b subunit 1	

SINE	selective inhibitor of nuclear export	selektivní inhibitor jaderného exportu
SRSF2	serine and arginine rich splicing factor 2	
TCR	T-cell receptor	receptor T-lymfocytů
TET2	ten-eleven translocation methylcytosine dioxygenase 2	
TL	T lymphocytes	T-lymfocyty
TRIP12	thyroid hormone receptor interactor 12	
UV	ultraviolet	ultrafialové
VAF	variant allele frequency	frekvence variantní alely
WB	western blot	western blot
WHO	World health organization	Světová zdravotnická organizace
wt	wild-type	přirozená forma

1 ÚVOD

Jadérko je dynamická jaderná bezmembránová organela, ve které dochází k syntéze a zpracování ribozomální RNA (rRNA; ribosomal RNA) a jejímu skládání do ribozomů a která se zároveň podílí na řízení buněčného cyklu a má významnou úlohu v regulaci buněčné odpovědi na stres. Mutace v jednom z jeho základních stavebních kamenů, nukleofosminu 1 (NPM1; nucleophosmin 1), se proto promítá do průběhu mnoha buněčných procesů a není překvapivé, že deregulace důležitých signálních drah má za následek nesprávný vývoj a proliferaci takto zasažené buňky a přispívá ke vzniku nádorového onemocnění.

Charakteristické mutace genu *NPM1* patří mezi nejčastější změny asociované s akutní myeloidní leukémií (AML; acute myeloid leukemia). Ačkoli je výskyt mutace NPM1 bez přítomnosti dalších aberací spojen s příznivou odpovědí na indukční chemoterapii, zejména pro starší pacienty s limitujícími komorbiditami tato intenzívní léčba není vhodná. Možnost zacílit leukemogenní mechanismy mutace *NPM1* tedy může přispět k úspěšnější terapii AML s mutovaným (mut; mutated) NPM1. Změna buněčné lokalizace NPM1 doprovázející tyto mutace a schopnost oligomerizace mutNPM1 pravděpodobně poskytují myeloidním buňkám proliferační výhodu. Následkem interakce mutované formy NPM1 s dalšími proteiny (např. tumor supresory p53 a p14Arf) a s molekulami přirozené formy (wt; wild-type) NPM1 se tyto interagující proteiny ocitají v cytoplazmě, tedy mimo původní místo svého působení. Využití oligomerizační domény NPM1 jako selektivního terapeutického cíle vyžaduje hlubší porozumění tvorbě oligomerních struktur a detailní charakteristiku interakčních vlastností proteinu NPM1. Tato práce se zaměřuje na studium oligomerizace přirozené i mutované formy NPM1 a představuje soubor metod pro stanovení oligomerních komplexů NPM1 v buněčných lyzátech i v živých buňkách. Současným tématem mnoha vědeckých studií jsou léčebné strategie ovlivňující interakční potenciál, lokalizaci či stabilitu NPM1. V rámci předkládané dizertační práce jsou diskutovány účinky léčiva NSC348884, které literatura popisuje jako účinný inhibitor oligomerizace NPM1.

2 SOUČASNÉ POZNATKY O NPM1 A JEHO ROLI V ONEMOCNĚNÍ AML

2.1 Struktura NPM1

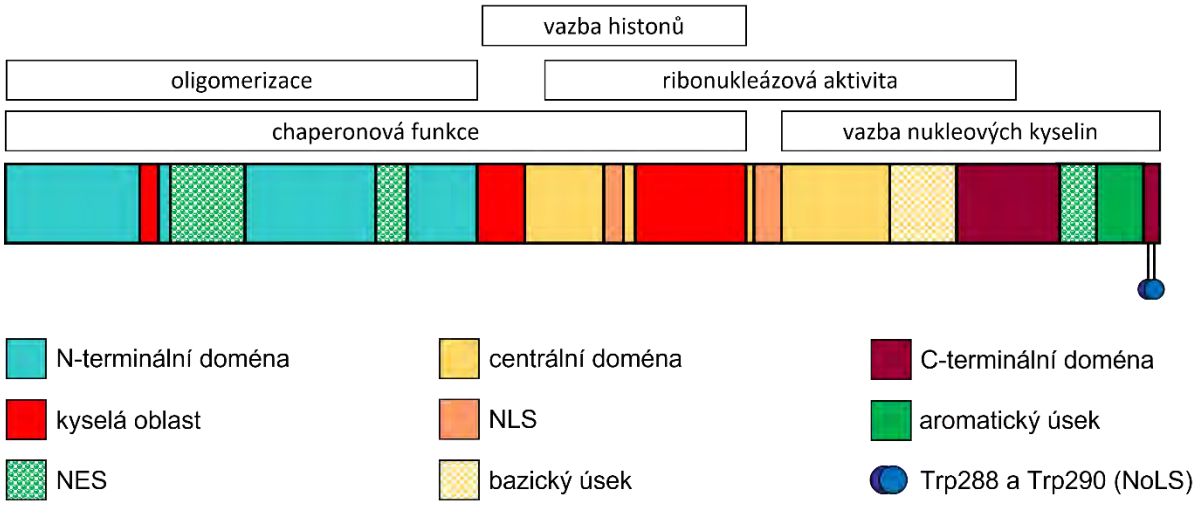
NPM1, někdy označovaný jako B23, je společně s nukleoplazminem 2, nukleoplazminem 3 a nukleoplazminovým proteinem u drozofily dNPL (z angl. *drosophila nucleoplasmin-like protein*) řazen do rodiny jaderných chaperonů z nukleofosmin/nukleoplazminové rodiny (Dutta et al., 2001; Eirín-López et al., 2006; Namboodiri et al., 2004, 2003). Některé studie do této rodiny řadí také nukleoplazminové proteiny u bezobratlých ANO39 a p62 (Namboodiri et al., 2003). Přes značné rozdíly v expresi, vnitrobuněčné lokalizaci a buněčné funkci vykazují N-koncové domény proteinů této rodiny vysokou sekvenční a strukturní podobnost (Frehlick et al., 2007; Namboodiri et al., 2003).

2.1.1 Gen *NPM1* a jeho transkripční varianty

Lidský gen *NPM1* o velikosti cca 23 kb je lokalizován v chromozomálním pruhu 5q35.1, obsahuje 12 exonů (neXtProt; NX_P06748) a kóduje přinejmenším 3 různé proteinové izoformy, přičemž počet, délka a nomenklatura alternativních transkriptů není v databázích jednotná. NPM1.1 (UniProt P06748-1) (u myší B23.1) představuje nejčastější transkripční variantu složenou z 11 exonů (1–9 a 11–12) a kóduje jadérkový protein o velikosti 32,6 kDa a délce 294 aminokyselin (AA; amino acid) exprimovaný ve všech typech tkání (Chang and Olson, 1990; Wang et al., 1993). Tato izoforma bývá v literatuře nejčastěji označována zkratkou NPM1, případně NPM. Proteinová izoforma NPM1.3 (UniProt P06748-3) (u myší B23.2) dlouhá 259 AA je kódována variantou s chybějícími exony 11 a 12, postrádá tedy doménu zodpovědnou za jadérkovou lokalizaci, a proto se vyskytuje také v nukleoplazmě (Colombo et al., 2006; Okuwaki et al., 2002). Jako jediná ze tří základních izoforem obsahuje exon 10, který nese stop kodon. Varianta NPM1.2 (UniProt P06748-2) se od varianty NPM1.1 liší delecí exonu 8, kóduje tedy kratší protein o velikosti 265 AA s nižší afinitou pro RNA, což způsobuje částečný přesun této izoformy do nukleoplazmy (Hisaoka et al., 2014). Biologická funkce a exprese NPM1.2 však není objasněna. Moje dizertační práce se zaměřuje na transkripční variantu NPM1.1, dále ji v textu uvádím pod zkratkou NPM1. Podrobná literární rešerše týkající se NPM1 je zpracována v review Nucleophosmin in leukemia: Consequences of anchor loss, jehož jsem spoluautorem (Brodská et al., 2019).

2.1.2 Struktura a funkce jednotlivých domén NPM1

NPM1 obsahuje několik částečně se překrývajících funkčních oblastí zodpovědných za řadu jeho buněčných aktivit (**Obr. 1**).



Obrázek 1: Funkční oblasti a signální sekvence NPM1.
NPM1 = nukleofosmin 1; NES = jaderný exportní signál; NLS = jaderný lokalizační signál; NoLS = jadérkový lokalizační signál.

N-koncová doména (NTD; N-terminal domain) vykazuje chaperonovou aktivitu, umožňuje většinu interakcí NPM1 s dalšími proteiny a je nezbytná pro jeho oligomerizaci (Hingorani et al., 2000; Mitrea et al., 2014; Szebeni and Olson, 1999). Je tvořena krátkým neuspořádaným úsekem methioninových zbytků, jejichž funkce zatím nebyla objasněna, a hydrofobním jádrem. To se formuje do osmi antiparalelních β-skládaných listů, které jsou stabilizovány hydrofobními a vodíkovými interakcemi a vytvářejí tzv. β-barel (Lee et al., 2007). Molekuly NPM1 tvoří pentamery vykazující asymetrické rozložení negativně nabitých funkčních skupin AA (Lee et al., 2007; Mitrea et al., 2014). AA 34–39 v NTD se označují jako první kyselá oblast A1 NPM1 (Lee et al., 2007). Rozsah NTD není v literatuře definován jednotně, nejčastěji se uvádí zhruba prvních 120 AA (Duan-Porter et al., 2014; Hingorani et al., 2000; Lee et al., 2007; Mitrea et al., 2014; Okuwaki, 2007; Qi et al., 2008).

Centrální doména NPM1 (120–242 AA (Di Matteo et al., 2016)) spolupracuje s NTD při plnění chaperonové funkce NPM1 (Colombo et al., 2011; Hingorani et al., 2000) a společně s C-koncovou doménou (CTD; C-terminal domain) zajišťuje ribonukleázovou aktivitu proteinu (Herrera et al., 1995; Hingorani et al., 2000). Jedná se o vnitřně neuspořádanou doménu zahrnující dvě kyselé oblasti, A2 (120–132 AA) (Gadad et al., 2011) a A3 (160–188 AA) (Grisendi et al., 2006), bohaté na kyselinu asparagovou a glutamovou. Tyto kyselé úseky umožňují svým záporným nábojem vazbu NPM1 k histonům, čímž podporují jeho funkci v sestavování nukleozomů a uspořádání chromatinu (Gadad et al., 2011; Swaminathan et al., 2005). Mezi A3 a CTD byl definován úsek 55 AA s převahou pozitivně nabitých postranních řetězců, které

pravděpodobně usnadňují interakci CTD NPM1 s nukleovými kyselinami (NA; nucleic acid) (Mitrea et al., 2018). Konkrétně neuspořádaný segment AA zbytků 225–242 s klíčovými lyziny Lys229 a Lys230 hraje nezastupitelnou roli v rozpoznání a vazbě úseků DNA bohatých na guanin sestavených do čtyřvláknových struktur známých jako G-kvadruplexy (Arcovito et al., 2014; Federici et al., 2010; Gallo et al., 2012) a v součinnosti s NTD udržuje stabilitu CTD (Marasco et al., 2013).

Jak bylo zmíněno v předchozím odstavci, u CTD (243–294 AA) (Grummitt et al., 2008) byla prokázána ribonukleázová aktivita a schopnost vázat NA (Dumbar et al., 1989; Wang et al., 1994). CTD je složena ze tří α-helixů stabilizovaných hydrofobním jádrem, které je tvořeno shlukem aromatických postranních řetězců konzervovaných AA Phe268, Tyr271, Phe276, Trp288 a Trp290. Řada lyzinových a argininových zbytků vyskytujících se v CTD uděluje této oblasti kladný náboj (Grummitt et al., 2008).

2.2 Vnitrobuněčná lokalizace NPM1

Většina molekul NPM1 se nachází v granulární složce jadérka (GC; granular component) (Feric et al., 2016; Michalik et al., 1981; Spector et al., 1984), nicméně signální sekvence v primární struktuře NPM1 umožňují jeho přemísťování mezi jadérkem, nukleoplazmou a cytoplazmou (Borer et al., 1989).

Součástí NTD jsou dvě signální sekvence pro přesun proteinu do cytoplazmy, tzv. jaderné exportní signály (NES; nuclear export signal), predikované v pozicích 42–61 AA (Yu et al., 2006) a 94–102 AA (Wang et al., 2005). NES je definován jako sekvence zhruba 8–15 AA obsahující charakteristické hydrofobní AA zbytky, z nichž bývá majoritně zastoupen leucin. Tyto motivy jsou rozpoznávány receptory pro jaderný export, z nichž k nejvýznamnějším patří exportin 1 známý také pod zkratkou CRM1 (z angl. *chromosomal region maintenance 1*) (Fung and Chook, 2014; la Cour et al., 2004; D. Xu et al., 2012). Exportní aktivita obou NES v NTD NPM1 však byla charakterizována jako hraniční až nedetekovatelná, a to zřejmě v důsledku jejich nevyhovující sekundární struktury (Arregi et al., 2015; Bolli et al., 2007). Od N-konce vzdálenější NES se uplatňuje v interakci NPM1 s centrozomem (Wang et al., 2005).

Kyselá oblast centrální domény NPM1, A3, je obklopena částmi sekvence (152–157 AA a 190–197 AA) (Grisendi et al., 2006) směřující protein do buněčného jádra, tzv. jaderným lokalizačním signálem (NLS; nuclear localization signal) (Hingorani et al., 2000).

Zásadní vlastnosti zajišťující jadérkovou lokalizaci NPM1 nese jeho C-koncová doména. Kromě důležitého jadérkového lokalizačního signálu (NoLS; nucleolar localization signal), jehož

klíčovou součástí jsou Trp288 a Trp290 (Alcalay et al., 2005; Falini et al., 2006b), je pro výskyt NPM1 v jadérku podstatná sekundární struktura CTD (zejména úsek sbalený do třetího α-helixu) (Scognamiglio et al., 2016). Za lokalizaci NPM1 v jadérku je zodpovědná i oblast karboxylového konce (CTD společně s přilehlým bazickým úsekem) s afinitou pro rRNA (Mitrea et al., 2018, 2016) a pro strukturu G-kvadruplexu (Federici et al., 2010; Gallo et al., 2012). Řetězec AA zbytků 268–278 obsahuje sekvenci, která by případně mohla sloužit jako NES, patrně se však jaderného exportu NPM1 neúčastní (Arregi et al., 2015).

Cílení NPM1 do jednotlivých buněčných kompartmentů je regulováno mimojiné interakcemi s mnoha proteiny, jejichž seznam je dostupný ve veřejných internetových databázích (https://thebiogrid.org/110929/summary/homo-sapiens/npm1.html). Např. řada jadérkových proteinů interaguje svými NoLS bohatými na arginin (tzv. R-motivy) s kyselými oblastmi NPM1, čímž posilují jeho akumulaci v jadérku (Mitrea et al., 2016).

Vnitrobuněčnou lokalizaci NPM1 ovlivňují také posttranslační modifikace (PTM; post-translational modifications), které se zároveň podílejí na regulaci jeho funkce, stability a interakčních vlastností. PTM mohou generovat konformační změny cílového proteinu, které v případě NPM1 úzce souvisejí s jeho oligomerizačním stavem a vnitrobuněčným umístěním (Mitrea et al., 2014). Zatímco rozpad NPM1 na monomery vyvolá jejich přesun do nukleoplazmy, oligomery NPM1 jsou soustředěny do buněčného jadérka (Enomoto et al., 2006; Jian et al., 2009). Jednu z nejvýznamnějších kovalentních modifikací NPM1 představuje fosforylace. Fosfátové skupiny vnášejí do molekuly NPM1 negativní náboj, čímž narušují stabilizační vliv kovových iontů na strukturu pentameru. Fosforylace Thr95 a Ser125 NPM1 způsobuje posun rovnováhy ve prospěch monomerů a zpřístupňuje tak pro kinázy další klíčová fosforylační místa, Ser48 a Ser88, jejichž fosforylace dále podporuje disociaci NPM1 do monomerního stavu (Mitrea et al., 2014). Nicméně dle jiné studie fosforylace Ser125 zprostředkovaná IκB-kinázou α stabilizuje strukturu oligomerů NPM1, jejichž zvýšená hladina podporuje asociaci NPM1 s centrozomem (Xia et al., 2013). Fosforylace Ser125 je proto důležitá pro udržení integrity genomu. Přesun NPM1 z jadérka indukuje také série fosforylací Thr199, Thr219, Thr234 a Thr237 v časné fázi mitózy, v jejímž důsledku pravděpodobně dochází k narušení afinity NPM1 k RNA (Hisaoka et al., 2014, 2010; Negi and Olson, 2006; Okuwaki et al., 2002). Deacetylace lyzinů Lys27 a Lys54 pomocí sirtuin 7 hraje významnou roli v reakci na genotoxický stres způsobený UV (UV; z angl. *ultraviolet*) zářením (Ianni et al., 2021). Naopak acetylace lyzinových zbytků Lys212, Lys215, Lys229, Lys230, Lys257 a Lys267 vyvolává

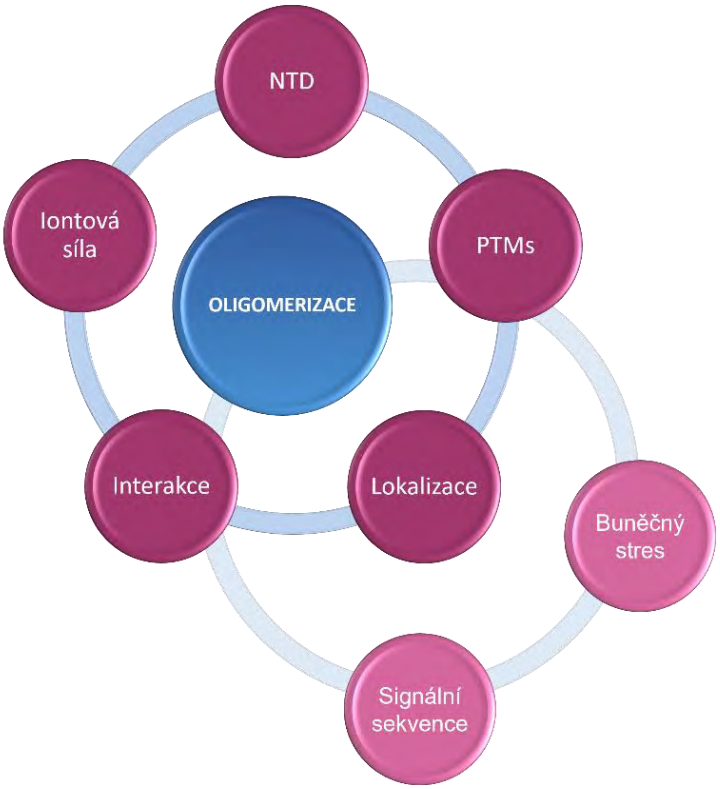
přemístění NPM1 z jadérka do nukleoplazmy, kde v transkripčně aktivních oblastech interaguje s RNA polymerázou II (RNAP; RNA polymerase) (Shandilya et al., 2009). V neposlední řadě je jak pro jadérkovou, tak pro centrozomální lokalizaci NPM1 klíčová sumoylace Lys263 (Liu et al., 2007).

Buněčný stres je často spojen s reorganizací struktury jadérka a se změnou skladby proteinů v něm obsažených (Boulon et al., 2010). Charakteristickou odezvou na genotoxický stres je uvolnění NPM1 z jadérka do nukleoplazmy. V reakci na oxidační zátěž podléhá NPM1 S-glutathionylaci na Cys275 (Yang et al., 2016), který je součástí CTD vázající NA (Wang et al., 1994). Tato PTM inhibuje schopnost CTD interagovat s NA a indukuje přesun NPM1 do nukleoplazmy, kde je následně spuštěna buněčná odpověď na stres řízená p53 (Yang et al., 2016). Translokaci NPM1 z jadérka do nukleoplazmy v případě postižení buňky nějakou formou stresu dokládá řada dalších publikací (Chan et al., 1988, 1987; Kalousek et al., 2005; Kodiha et al., 2011; Kurki et al., 2004; B. Y.-M. Yung et al., 1985; Yung et al., 1986; B. Y. M. Yung et al., 1985). Je zaznamenán také přesun NPM1 do cytosolu, kde tvorbou ribonukleoproteinového komplexu s hnRNPU (z angl. *heterogeneous nuclear ribonucleoprotein U*) a hnRNPA1 (z angl. *heterogeneous nuclear ribonucleoprotein A1*) napomáhal přežít buňkám vystaveným vlivu Aktinomycinu D (ActD; Actinomycin D) (Yao et al., 2010).

2.3 Oligomerizace NPM1

Modulace oligomerního a monomerního stavu proteinu představuje důležitý regulační mechanismus jeho aktivity. NPM1 se v buňkách standardně vyskytuje převážně v oligomerech, které vznikají interakcí N-koncových domén jednotlivých molekul (Hingorani et al., 2000). Tématu oligomerizace NPM1 se věnuje řada studií zkoumajících především esenciální oblasti NPM1 zodpovědné za tvorbu oligomerů a také faktory ovlivňující tento proces (**Obr. 2**). Již delece prvních 24 AA NPM1 měla za následek ztrátu schopnosti takto zkráceného proteinu interagovat s endogenním NPM1 a vedla k jeho částečnému uvolnění do nukleoplazmy (Enomoto et al., 2006). V případě rozsáhlejších delecí NTD je taktéž popsána inhibice oligomerizace a přemístění těchto kratších variant NPM1 z jadérka do nukleoplazmy (Enomoto et al., 2006). Naše experimenty však ukázaly, že ačkoliv je schopnost interakce takto mutovaných proteinů s wtNPM1 významně snížena, není zcela inhibována (Šašinková et al., 2021). Dle studie Enomoto *et al.* pak odstranění posledních 102 AA v CTD NPM1 posílilo interakci tohoto fragmentu s endogenním NPM1 a zároveň jeho majoritní frakce zůstala

lokalizována v jadérku (Enomoto et al., 2006). Dalším klíčovým úsekem pro oligomerizaci NPM1 je oblast zahrnující konzervované AA zbytky Leu102, Gly105, Ser106, Gly107 a Pro108 (Dutta et al., 2001; Enomoto et al., 2006). Jejich mutace (vyjma NPM1 p.Ser106Ala) vedly k destabilizaci oligomerů NPM1 a k rozptýlení těchto mutantů do nukleoplazmy (Enomoto et al., 2006). V sestavování a stabilitě NPM1 oligomerů hrají důležitou roli také AA zbytky Tyr67 v β-vlásence spojující β4 a β5-skládaný list oligomerizační domény (Duan-Porter et al., 2014) a Cys21, který je navíc nezbytný i pro chaperonovou aktivitu NPM1 (Prinos et al., 2011). Substituce Cys21 za aromatickou hydrofobní AA (Phe, Trp) dle publikace Prinos *et al.* inhibuje tvorbu NPM1 pentamerů (Prinos et al., 2011). Novější studie Holoubek *et al.* využívající navíc pozorování v živých buňkách založené na časově rozlišené fluorescenční mikroskopii potvrzuje vliv této substituce na stabilitu NPM1 oligomeru v buněčných lyzátech, zároveň však popisuje schopnost mutované formy p.Cys21Phe proteinu NPM1 interagovat jak s variantou p.Cys21Phe, tak s přirozeným NPM1 (Holoubek et al., 2018).



Obrázek 2: Schéma zobrazující faktory ovlivňující oligomerizaci a lokalizaci NPM1. Oligomerizační stav NPM1 může být ovlivněn jeho interakcemi s jinými proteiny, iontovou silou prostředí, aminokyselinovými sekvencemi v N-koncové doméně, posttranslačními modifikacemi či jeho vnitrobuněčnou lokalizací. Podobně o vnitrobuněčném umístění NPM1 rozhodují interakce a oligomerizace molekul NPM1, posttranslační modifikace, přítomnost sekvencí jadérkové a jádrové lokalizace a jaderného exportu či určitá forma buněčného stresu. NPM1 = nukleofosmin 1; NTD = N-terminální doména; PTMs = posttranslační modifikace.

S oligomerizačním stavem NPM1 souvisí kromě vnitrobuněčné lokalizace také stabilita proteinu. NPM1 patří mezi stabilnější bílkoviny s poločasem rozpadu větším než 24 hodin (Itahana et al., 2003). Různě rozsáhlé delece v NTD NPM1 a dvojitá substituce p.Leu102Ala + p.Gly105Ala narušily nejen oligomerizační schopnost proteinu, ale také jeho životnost, která byla snížena zhruba na 10 hodin. Naproti tomu fragment s chybějícím úsekem 102 AA na C-konci NPM1 vykazoval srovnatelný poločas rozpadu jako wtNPM1 (Enomoto et al., 2006). Lze tedy shrnout, že NPM1 nevázaný v oligomerech podléhá masivní relokalizaci z jadérka do nukleoplazmy, kde je zřejmě účinněji degradován.

Oligomerizace NPM1 může být ovlivněna jeho interakčními partnery. V experimentech s peptidy odvozenými od N-konce tumor supresoru p14Arf, známého NPM1-vazebného proteinu, byla demonstrována schopnost těchto fragmentů stabilizovat NPM1 v jeho pentamerickém uspořádání (Banerjee et al., 2016). Dalším faktorem působícím na oligomerizaci NPM1 je iontová síla. V prostředí s nízkou iontovou silou a bez dostupných mono- a divalentních kationtů se NPM1 nachází zejména ve formě monomerů. Jejich spojení do oligomerních komplexů lze naopak podnítit působením vyšší iontové síly a přítomností jednomocných a dvoumocných kationtů, které odstiňují odpudivé elektrostatické interakce mezi kyselými oblastmi A1 a A2 jednotlivých NPM1 monomerů (Herrera et al., 1996; Mitrea et al., 2014). Jak bylo uvedeno již v kapitole 2.2 Vnitrobuněčná lokalizace NPM1, úlohu v tvorbě či rozpadu oligomerů sehrává rovněž řada PTM ovlivňujících konformaci NPM1 (Mitrea et al., 2014).

Zásah do oligomerizace NPM1 je jedním z nástrojů vhodných pro vývoj nových terapeutických látek užívaných pro léčbu hematologických malignit. O tomto přístupu bude pojednáno v kapitole 2.6.1.3 Cílená léčba AML s mutNPM1.

2.4 Funkce NPM1

Gen *NPM1* kóduje multifunkční fosfoprotein s buněčnou aktivitou převážně v jadérku, nicméně schopnost NPM1 přemísťovat se z jádra do cytoplazmy a naopak rozšiřuje jeho pole působnosti i mimo jadérko.

Chaperonová aktivita NPM1 zahrnuje histonové i nehistonové cíle. Afinita NPM1 k histonům usnadňuje jejich zabudování do nukleozomu, v souvislosti s acetylací svého C-konce navozuje NPM1 rozvolnění nukleozomové struktury a je zapojen do procesů udržujících a měnících strukturu chromatinu (Okuwaki et al., 2001a, 2001b; Swaminathan et al., 2005). Jako molekulární chaperon zabraňuje agregaci proteinů, udržuje jejich

enzymatickou aktivitu během teplotní denaturace, umožňuje renaturaci denaturovaných bílkovin (Szebeni and Olson, 1999) a zamezuje nevratné agregaci špatně sbalených proteinů migrujících do jadérka v reakci na tepelný stres (Frottin et al., 2019).

Schopnost NPM1 regulovat transkripci genů pro rRNA odráží jeho funkci histonového chaperonu (Murano et al., 2008). Zároveň společně s jeho ribonukleázovou aktivitou (Herrera et al., 1995), účastí v sestavování ribozomálních podjednotek (Schmidt-Zachmann et al., 1987) a v jejich exportu do cytoplazmy (Borer et al., 1989; Yu et al., 2006) poukazuje na nezbytnou úlohu NPM1 v biogenezi ribozomů.

Vedle exportu ribozomálních podjednotek může NPM1 zprostředkovávat také jadernou či jadérkovou lokalizaci malých proteinů jakými jsou např. Rev (Fankhauser et al., 1991; Szebeni et al., 1997) a Tat (z angl. *trans-activator of transcription*) (Li, 1997) viru lidské imunodeficience (HIV; human immunodeficiency virus), Rex lidského T-buněčného lymfotropního viru typu I (Adachi et al., 1993), kapsidový protein viru japonské encefalitidy (Tsuda et al., 2006), p120 (Valdez et al., 1994) či GADD45α (z angl. *growth arrest and DNA damage inducible gene 45 alpha*) (Gao et al., 2005). Uvádí se, že i jeden z nejčetnějších jadérkových proteinů nukleolin (NCL; nucleolin) je udržován v jadérku kromě jiného díky interakci s NPM1 (Li et al., 1996).

NPM1 je zapojený do reparačních mechanismů opravujících poškození DNA pomocí bázové (Poletto et al., 2014) a nukleotidové (Wu et al., 2002) excizní opravy, účastní se duplikace centrozomu (Okuda et al., 2000; Wang et al., 2005), replikace DNA (Okuwaki et al., 2001a; Takemura et al., 1999, 1994) a transkripce katalyzované RNAP I (Bergstralh et al., 2007; Lessard et al., 2010; Murano et al., 2008) a RNAP II (Gurumurthy et al., 2008; Swaminathan et al., 2005). Všemi těmito aktivitami NPM1 přispívá k udržování stability genomu.

NPM1 byl identifikován jako klíčový protein v organizaci jadérka. Interakce pentamerů NPM1, proteinů s R-motivy a rRNA byly popsány jako nezbytné pro fázovou separaci jadérka, v jejímž důsledku lze pozorovat vrstevnatou jadérkovou strukturu (Feric et al., 2016; Mitrea et al., 2016).

Dále se NPM1 v komplexu s malými jadérkovými RNA a fibrilarinem (FBL; fibrillarin) účastní metylace ribózy rRNA na 2‘-OH skupině (Nachmani et al., 2019) – jedné z nejčastějších posttranskripčních modifikací rRNA, která hraje důležitou roli v přesnosti a účinnosti translace (Decatur and Fournier, 2002; Sharma and Lafontaine, 2015).

V neposlední řadě je NPM1 schopen regulovat průběh buněčného cyklu a apoptózu pomocí četných interakcí s dalšími proteiny zapojenými do těchto procesů, což je podrobněji popsáno v následující kapitole.

2.5 Interakce NPM1

NPM1 disponuje rozmanitou skladbou vazebných partnerů, které jsou přímo či nepřímo zapojeny do řady buněčných procesů regulovaných NPM1. Většina interakcí je uskutečněna prostřednictvím NTD NPM1, zřídka je zapojena střední a C-koncová část NPM1 (např. pro vazbu NCL (Li et al., 1996), p53 (Colombo et al., 2002; Lambert and Buckle, 2006), p120 (Valdez et al., 1994), Tat (Li, 1997), Rex (Adachi et al., 1993)). V souvislosti s hojným výskytem NPM1 v GC jadérka, v níž probíhá sestavování ribozomálních podjednotek z ribozomálních proteinů a rRNA, byla predikována a následně potvrzena jeho schopnost vázat NA (Dumbar et al., 1989; Wang et al., 1994). NPM1 interaguje s RNA i DNA (s vazebnou preferencí pro jednovláknové řetězce DNA oproti struktuře dvouvláknové DNA) (Dumbar et al., 1989; Wang et al., 1994). Mezi významné proteiny a peptidy interagující s NPM1 patří ribozomální proteiny RPLP5 (Yu et al., 2006), RPS9 (Lindström and Zhang, 2008) a RPL23 (Wanzel et al., 2008), neribozomální jadérkový protein NCL (Li et al., 1996; Liu and Yung, 1999) a dle kolokalizačních (Amin et al., 2008) a imunoprecipitačních (Holoubek 2021) analýz také FBL, histony H1, H2A, H2B, H3 a H4 (Gadad et al., 2011; Okuwaki et al., 2001b; Swaminathan et al., 2005), proteinkinázy Aurora A (Reboutier et al., 2012) a B (Shandilya et al., 2014) zapojené do procesu buněčného dělení, virové proteiny Tat (Li, 1997) a Rev (Fankhauser et al., 1991) viru HIV, kapsidový protein Cp149 hepatitidy B (Lee et al., 2009) a kapsidové bazické proteiny adenoviru (Okuwaki et al., 2001a). Dále pak také apurinová/apyrimidinová endonukleáza 1 (APE1; apurinic/apyrimidinic endonuclease 1), jejíž aktivita je stimulovaná vazbou NPM1 na abazických místech dvouvláknové DNA (Vascotto et al., 2009), transkripční faktory C-MYC (z angl. *cellular myelocytomatosis*) (Li et al., 2008), NF-κB (z angl. *nuclear factor kappa-light-chain-enhancer of activated B cells*) (Dhar et al., 2004), MEF (z angl. *myeloid Elf-1-like factor*) (Ando et al., 2013), STAT1 (z angl. *signal transducer and activator of transcription 1*) a IRF1 (z angl. *interferon regulatory factor 1*) zapojené do cytokinové signalizace (Abe et al., 2018), regulátor myeloidní diferenciace PU.1 (Gu et al., 2018) a tumor supresor p53 (Colombo et al., 2002). Posledně uvedený je součástí komplexu několika vzájemně se ovlivňujících proteinů regulujících apoptózu, jehož významnými členy jsou rovněž p14Arf a MDM2 ((z angl. *mouse double minute 2*), v lidských

buňkách také označovaný jako HDM2 (z angl. *human homolog of mouse double minute 2*)). O proteinech této interakční sítě je pojednáno v následujících odstavcích.

2.5.1 p53

Tumor supresorový protein p53, označovaný jako strážce genomu, funguje především jako transkripční faktor regulující expresi cílových genů v reakci na buněčný stres a poškození DNA (Feroz and Sheikh, 2020). Řada aktivit p53 je však uskutečňována i mimo jádro buňky prostřednictvím jeho interakcí s mimojadernými proteiny (Comel et al., 2014). Zastavením buněčného cyklu umožňujícím buňce reparaci DNA, navozením senescence omezující šíření starých nebo defektních buněk či indukcí apoptózy p53 významně potlačuje nádorové bujení poškozených buněk (Levine, 1997). Mezi důležité regulátory stability a aktivity p53 patří NPM1. NPM1 může v závislosti na fyziologických stimulech, aktivované signální dráze, buněčném typu a dle svého množství a lokalizace v buňce působit jako proapoptotický i protiapoptotický protein (Colombo et al., 2011). Zvýšená exprese NPM1 vyvolává přesun p53 do jadérka, kde NPM1 přímou vazbou oblasti 186–259 AA (respektive 249–262 AA (Lambert and Buckle, 2006)) k p53 zvyšuje jeho stabilitu a udržuje ho v aktivním stavu (Colombo et al., 2002). Byly identifikovány různé oblasti p53 zodpovědné za interakci s NPM1. Jedna studie označuje jako důležitou pro tuto interakci dvojici oblastí, součásti DNA-vazebné domény (175–196 AA) a oligomerizační domény p53 (343–363 AA) (Lambert and Buckle, 2006), zatímco jiná studie mapuje NPM1 vazebnou doménu na N-konci p53 (Maiguel et al., 2004). Naopak protiapoptotické působení NPM1 zaznamenává řada vědeckých prací, z nichž vyplývá, že nadměrná exprese NPM1 přispívá k vyšší odolnosti buněk vůči oxidačnímu stresu (Dhar and St. Clair, 2009; Li et al., 2006) a stresu vyvolanému UV zářením (Maiguel et al., 2004; Wu et al., 2002), hypoxií (Li et al., 2004) či zesílenou expresí onkogenů (Li et al., 2007). Předčasnému rozvoji apoptózy způsobené UV zářením zamezuje NPM1 zvýšením mezní hodnoty UV energie, při které je indukována fosforylace p53 na Ser15 vedoucí k jeho aktivaci (Maiguel et al., 2004). Tímto mechanismem a dále stimulací opravných procesů DNA a exprese svíracího proteinu PCNA (z angl. *proliferating cell nuclear antigen*) (Wu et al., 2002) přispívá vysoká hladina NPM1 v buňkách k jejich odolnosti vůči zastavení buněčného růstu a apoptóze spuštěné UV zářením, čímž však může usnadnit rozvoj a progresi neoplázie. NPM1 blokuje fosforylaci p53 na Ser15 také v buňkách vystavených hypoxickému prostředí, v nichž je stabilizován hypoxií indukovaný transkripční faktor 1 (HIF-1; hypoxia-inducible factor 1) aktivující expresi NPM1 (Li et al., 2004). Zvýšená hladina NPM1 v reakci na nedostatek kyslíku v nádorových buňkách tak zesiluje jejich

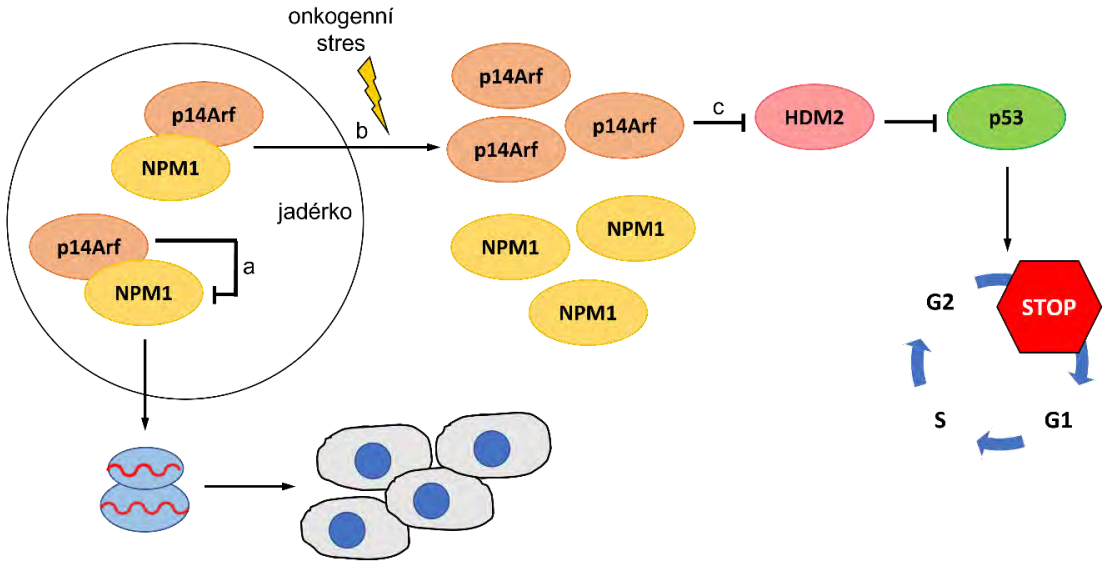
odolnost vůči hypoxickému stresu a zabraňuje apoptóze (Li et al., 2004). Nadměrná exprese NPM1 potlačila akumulaci fosforylované formy p53 na Ser15 také v buňkách s posílenou expresí C-MYC stimulujícího aktivitu p53 (Li et al., 2007). Mezi významné proteiny, jejichž exprese je regulována p53, patří inhibitor cyklin-dependentních kináz, p21, jenž zastavuje progresi buněčného cyklu a tlumí replikaci DNA (Luo et al., 1995), či proapoptotický protein BAX (z angl. *B-cell lymphoma-2-associated X protein*) (Toshiyuki and Reed, 1995). Oba tyto proteiny byly identifikovány také jako vazební partneři NPM1 (Kerr et al., 2007; Xiao et al., 2009).

2.5.2 p14Arf

p14ARF, protein lokalizovaný zejména v jadérku, představuje klíčový aktivátor dráhy p53. V odpověď na buněčný stres je p14Arf uvolněn do nukleoplazmy, kde svojí vazbou na specifickou E3 ubikvitin-ligázu pro p53, MDM2 (Pomerantz et al., 1998; Zhang et al., 1998), blokuje její ubikvitinylační aktivitu (Honda, 1999) nebo ji přesouvá z nukleoplazmy do jadérka, čímž zamezuje interakci MDM2 s p53 (Weber et al., 1999). Díky tomu dochází ke stabilizaci a akumulaci p53 v buněčných jádrech (Chen et al., 2005; Zhang et al., 1998). Mimoto protein p14Arf vykonává řadu tumor-supresorových funkcí nezávislých na p53, jako je zastavení buněčného cyklu skrze interakci s transkripčním faktorem E2F-1 (z angl. *E2F transcription factor 1*) (Eymin et al., 2001) či prostřednictvím aktivace signální dráhy zprostředkované proteiny ATM (z angl. *ataxia telangiectasia-mutated*), ATR (z angl. *ataxia telangiectasia and Rad3-related*) a CHK (z angl. *checkpoint kinase*) (Eymin et al., 2006). Podílí se také na inhibici buněčného růstu a biogeneze ribozomů snížením hladiny NPM1 (Itahana et al., 2003) (podrobněji popsáno níže) nebo skrze nukleoplazmatickou akumulaci transkripčního terminační faktoru TTF-I (z angl. *transcription termination factor*) ovlivňujícího transkripci rRNA genů RNAP I (Lessard et al., 2010). Dále p14Arf přispívá k regulaci exprese genu XPC (z angl. x*eroderma pigmentosum, complementation group C*) účastnícího se nukleotidové excizní opravy DNA (Dominguez-Brauer et al., 2009), k aktivaci procesů autofágie (Abida and Gu, 2008; Reef et al., 2006) nebo k sumoylaci p14Arf vazebných partnerů HDM2 (Xirodimas et al., 2002), WRN (z angl. *Werner syndrome*) helikázy (Woods et al., 2004), E2F-1, HIF-1α, TATA vazebného proteinu, p120E4F (Rizos et al., 2005) a NPM1 (Tago et al., 2005).

Pro zajištění stabilizace a jadérkové lokalizace p14Arf je nezbytná jeho interakce s NPM1 (Colombo et al., 2005; Korgaonkar et al., 2005). N-koncová oblast NPM1 (konkrétně 100–116 AA) (Enomoto et al., 2006; Itahana et al., 2003) asociuje s doménou p14Arf

zodpovědnou za jadérkovou lokalizaci proteinu a jeho interakci s HDM2 (1–14 a 26–37 AA) (Korgaonkar et al., 2005) za vzniku supramolekulárních komplexů o velikosti 2–5 MDa (Bertwistle et al., 2004), čímž je potlačena aktivace p53 (Korgaonkar et al., 2005). Během odpovědi na buněčný stres je p14Arf uvolněn z jadérkového komplexu s NPM1 do nukleoplazmy, kde rozpoznává a váže HDM2 a tím stabilizuje p53 (Korgaonkar et al., 2005) (**Obr. 3**). Zatímco v buňkách se sníženou hladinou NPM1 byla detekována zvýšená nukleoplazmatická frakce p14Arf, nadměrná exprese NPM1 podpořila akumulaci p14Arf v jadérku (Korgaonkar et al., 2005). Rakovinné buňky různých typů nádorů často vykazují zvýšenou expresi NPM1 (Gimenez et al., 2010; Nozawa et al., 1996; Pianta et al., 2010; Shields et al., 1997; Skaar et al., 1998; Subong et al., 1999; Tsui et al., 2004; Yun et al., 2007), prostřednictvím něhož je p14Arf zadržen v jadérku a nemůže tak stimulovat buněčnou odpověď závislou na p53. V případě, že tyto buňky nesou nemutované formy p53 a p14Arf, vývoj a použití inhibitorů interakce NPM1-p14Arf nabízí slibnou možnost molekulárně cílené terapie (Korgaonkar et al., 2005). Na druhou stranu nízká koncentrace NPM1 nezajistí dostatečnou stabilitu p14Arf, což též může zvyšovat náchylnost buňky k maligní transformaci (Colombo et al., 2005; Korgaonkar et al., 2005).



Obrázek 3: Komplexy NPM1 a p14Arf a jejich vliv na průběh buněčného cyklu. NPM1 interaguje s p14Arf v jadérku za vniku vysokomolekulárních komplexů. NPM1 stabilizuje p14Arf a zprostředkovává jeho jadérkovou lokalizaci. **a)** p14Arf potlačuje aktivitu NPM1 a snižuje jeho hladinu v buňce, což se projeví sníženou biogenezí ribozomů a následně utlumenou buněčnou proliferací. **b)** V reakci na onkogenní stres dochází k rozpadu komplexu NPM1-p14Arf a k uvolnění obou proteinů do nukleoplazmy. **c)** V nukleoplazmě p14Arf vazbou k HDM2 stabilizuje p53 a ten způsobí zastavení buněčného cyklu. NPM1 = nukleofosmin 1; HDM2 = human homolog of mouse double minute 2.

Vzájemná interakce proteinů NPM1-p14Arf neovlivňuje pouze aktivitu p14Arf, ale také hladinu NPM1 v buňce. p14Arf způsobuje úbytek NPM1 prostřednictvím jeho ubikvitinylace a následné degradace v 26S proteazomu (Itahana et al., 2003). Protože NPM1 štěpí vnitřní transkribovaný mezerník ITS2 (z angl. *internal transcribed spacer*) oddělující geny pro 5.8S rRNA a 28S rRNA (Savkur, 1998), snížená hladina NPM1 vede k utlumení biogeneze ribozomů (Itahana et al., 2003). Endoribonukleázová aktivita NPM1 může být inhibována také skrze jeho navázání do komplexů p14Arf v jadérku, kde je NPM1 vůči degradaci zprostředkované p14Arf odolnější než NPM1 lokalizovaný v nukleoplazmě (Itahana et al., 2003). p14Arf interagující s NPM1 tedy představuje důležitý článek v inhibici syntézy ribozomů.

2.5.3 MDM2

Jak bylo zmíněno v předchozí kapitole, protoonkogen MDM2 (v lidských buňkách označovaný jako HDM2) funguje jako důležitý negativní regulátor p53 (Oliner et al., 1992). Tato negativní regulace je uskutečňována přímou vazbou MDM2 k transaktivační doméně p53 (Momand et al., 1992) nebo skrze ubikvitin-ligázovou aktivitu MDM2, která katalyzuje přenos ubikvitinu na p53. Monoubikvitinylace stimuluje přesun p53 do cytoplazmy, kde je jeho transkripční aktivita potlačena (Li et al., 2003), série ubikvitinů je pak signálem pro proteazomální degradaci p53 (Honda et al., 1997; Kubbutat et al., 1997). Zároveň je ale transkripce MDM2 závislá na p53, vzniká tedy smyčka regulující současně expresi MDM2 a aktivitu p53 (Wu et al., 1993). Ačkoli studie Maiguel *et al.* popisuje, že NPM1 v reakci na poškození DNA způsobené UV zářením tlumí transkripční aktivitu p53 (Maiguel et al., 2004), jiní autoři dokládají opačný účinek NPM1. Dle Colombo *et al.* a Kurki *et al.* UV záření vyvolává rychlý přesun NPM1 z jadérka do nukleoplazmy, kde dochází k jeho interakci s HDM2 a následně k akumulaci a stabilizaci p53 (Colombo et al., 2002; Kurki et al., 2004). NPM1 se skrze interakci s HDM2 podílí na regulaci bazální aktivity p53 také v kontrolních buňkách, které nebyly vystaveny stresu (Kurki et al., 2004).

2.5.4 NCL

NCL patří k hojně zastoupeným neribozomálním proteinům jadérka, kde je koncentrován do jeho husté fibrilární složky obklopující fibrilární centra a do GC (Brodská et al., 2016a; Smetana et al., 1984; Tajrishi et al., 2011). Ačkoli se nejvíce vyskytuje v jadérku, malá frakce je přítomna také v cytoplazmě, nukleoplazmě a na povrchu buňky (Scott and Oeffinger, 2016). NCL je potřebný pro biogenezi ribozomů a syntézu rRNA (Abadía-Molina et al., 1998; Bouche et al., 1984; Bourbon et al., 1983; Egyhazi et al., 1988; Ginisty et al., 1998;

Roger et al., 2003; Sipos and Olson, 1991), pro správnou segregaci chromozomů, organizaci jadérka a dělícího vřeténka (Ma et al., 2007; Ugrinova et al., 2007), účastní se buněčných oprav DNA (Goldstein et al., 2013; Kawamura et al., 2019; Kobayashi et al., 2012), regulace replikace (Daniely and Borowiec, 2000; Kim et al., 2005; Nasirudin et al., 2005; Tuteja et al., 1995; Wang et al., 2001) a transkripce (Bouche et al., 1984; Grinstein et al., 2002; Hanakahi et al., 1997; Huddleson et al., 2006; Roger et al., 2002; Yang et al., 1994), zajišťuje schopnost sebeobnovy a proliferace embryonálních kmenových buněk (Li et al., 2009; Yang et al., 2011), plní funkci histonového chaperonu (Angelov et al., 2006) a je možné, že jako protein pohybující se mezi jádrem a cytoplazmou (Borer et al., 1989; Schmidt-Zachmann et al., 1993) je zapojen také do nukleo-cytoplazmatického transportu ribozomálních komponent (Bouvet et al., 1998).

Jadérkovou lokalizaci NCL zprostředkovává mimo jiné interakce s NPM1 (Li et al., 1996). Vazebná oblast NPM1 pro NCL zahrnuje AA 187–245 (Li et al., 1996). Oba proteiny ovlivňují řadu buněčných pochodů spojených s biogenezí ribozomů, apoptózou, transkripcí, replikací a opravnými procesy DNA (Scott and Oeffinger, 2016) a částečně sdílejí také spektrum vazebných partnerů účinkujících v těchto procesech (https://thebiogrid.org/110929/summary/homo-sapiens/npm1.html; https://thebiogrid.org/110771/summary/homo-sapiens/ncl.html).

2.6 Role NPM1 v onkologických onemocněních

U řady solidních nádorů, např. tlustého střeva a konečníku (Nozawa et al., 1996), vaječníků (Shields et al., 1997), prsu (Skaar et al., 1998), močového měchýře (Tsui et al., 2004), prostaty (Subong et al., 1999), jater (Yun et al., 2007), štítné žlázy (Pianta et al., 2010) či astrocytomu (Gimenez et al., 2010), často dochází ke zvýšené expresi NPM1, čehož může být využito v určení základní diagnózy. Vysoká hladina proteinu NPM1 v buňkách solidních nádorů je podle metaanalýzy z roku 2018 významným parametrem nepříznivé prognózy (Chen et al., 2018). V případě nádoru žaludku byla pozorována heterogenní exprese NPM1 (Leal et al., 2014; Tanaka et al., 1992; Zhou et al., 2016).

V různých typech myeloidních a lymfoidních malignit se objevují charakteristické chromozomální translokace genu *NPM1*. U promyelocytární leukémie je jednou z variantních translokací t(5;17), která vede ke vzniku fúzního proteinu NPM1 s receptorem kyseliny retinové alfa (RARα; retinoic acid receptor alpha) (Redner et al., 1996). U pacientů s anaplastickým velkobuněčným lymfomem a s expresí anaplastické lymfomové kinázy (ALK; anaplastic lymphoma kinase) bývá jednou z nejčastějších přestaveb t(2;5) zahrnující geny

NPM1 a *ALK* (Morris et al., 1994). Translokace t(3;5) vytvářející fúzní gen *NPM1::MLF1* (z angl. *myeloid leukemia factor 1*) (Falini et al., 2006a; Yoneda-Kato et al., 1996) a t(5;18) způsobující fúzi genů *NPM1* a *HAUS1* (z angl. *HAUS augmin-like complex, subunit 1*) (Campregher et al., 2016) jsou asociovány s AML a myelodysplastickým syndromem (MDS; myelodysplastic syndrome). Nově charakterizované translokace genu *NPM1* představují t(5;10) NPM1::RPP30, t(5;18) NPM1::SETBP1 a t(5;6) NPM1::CCDC28A (Martelli et al., 2021; Martelli, 2018). Proteinové produkty uvedených fúzních genů obsahují N-koncovou doménu původního NPM1 a jsou v různé míře lokalizovány v nukleoplazmě a/nebo exportovány do cytoplazmy.

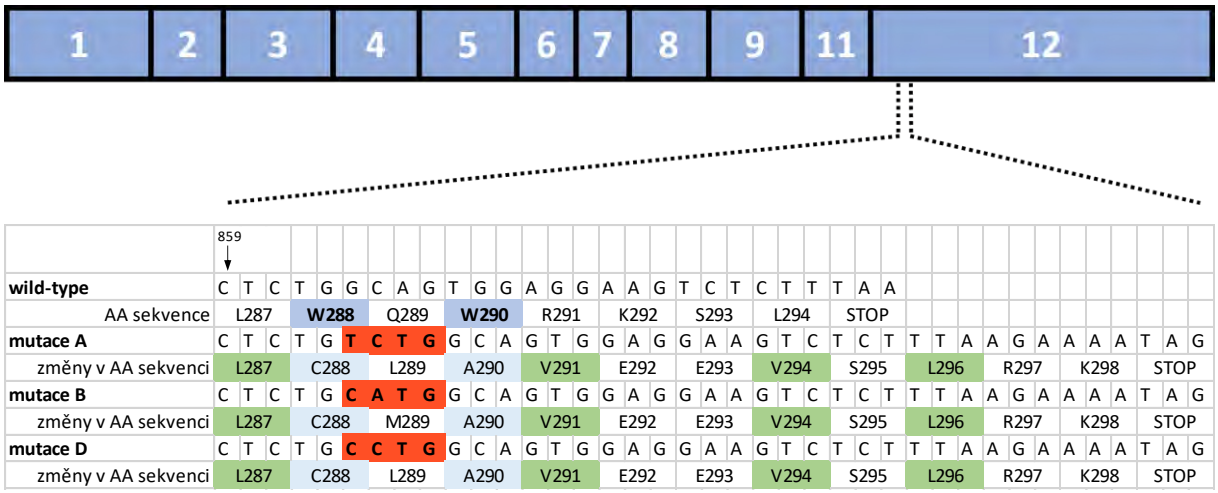
Mezi časté cytogenetické abnormality u MDS a AML dále patří intersticiální delece dlouhého ramene chromozomu 5 (del(5q))(Grimwade et al., 2001; Nimer and Golde, 1987; Solé et al., 2000; Tasaka et al., 2008), která se vzácněji objevuje i u akutní lymfoblastické leukémie (Berger et al., 1992; La Starza et al., 2016) či myeloproliferativních onemocnění (Bacher et al., 2009). U pokročilých forem MDS s del(5q) byla pozorována snížená exprese *NPM1* (Pellagatti et al., 2011) a u více než 40 % pacientů s vysoce rizikovým MDS či AML s komplexním karyotypem a monozomií chromozomu 5 byla patrná haploinsuficience NPM1 (La Starza et al., 2010).

Charakteristické mutace genu *NPM1* jsou uváděny jako nejčastější mutace u AML s normálním karyotypem (NK; normal karyotype), přičemž se jedná o klinicky významný faktor v odpovědi na léčbu a přežití (Falini et al., 2005). NPM1 mutacím vyskytujícím se u AML (AML s mutNPM1) se podrobněji věnuji v následující podkapitole. Ačkoli se nález NPM1 mutace typicky váže s onemocněním AML, může být zřídka prokázán také u MDS (4,4 %) (Bains et al., 2011) či chronické myelomonocytární leukémie (< 5 %) řazených mezi myeloidní neoplázie (MNs; myeloid neoplasms) (Peng et al., 2016; Vallapureddy et al., 2017). Nová studie revidující MNs s mutovaným NPM1 a s blasty zmnoženými pod 20 % však rozporuje vyčlenění této skupiny onemocnění jako samostatné klinické entity (Forghieri et al., 2020). U těchto pacientů bývá podobně jako u pacientů s AML s mutNPM1 nalezen NK, negativita pro antigen CD34, dobrá odpověď na léčbu intenzivní chemoterapií a velmi častá progrese do AML.

2.6.1 AML s mutNPM1

Specifické NPM1 mutace se vyskytují zhruba u 30 % nově diagnostikovaných dospělých AML, převážně s NK (Cancer Genome Atlas Research Network et al., 2013; Falini et al., 2005; Papaemmanuil et al., 2016). Na genové úrovni se obvykle jedná o inzerci 4 bp mezi nukleotidy 863 a 864 v exonu 12 genu *NPM1* (**Obr. 4**) (Chou et al., 2006; Falini et al., 2005; Schnittger et

al., 2005; Thiede et al., 2006) (výjimečně je zjištěna komplexní mutace sestávající z delece a inzerce (Bacher et al., 2014; Duployez et al., 2018; Ivey et al., 2016; Jeziskova et al., 2017; Thiede et al., 2006)). To má za následek posun čtecího rámce a vznik alternativního stop kodonu. Tyto chyby jsou zřejmě výsledkem nepřesné replikace DNA katalyzované terminální deoxynukleotidyltransferázou (Borrow et al., 2019). Výsledný proteinový produkt mutNPM1 má nahrazenu sekvenci nejčastěji posledních 5–7 AA ((WQ)WRKSL) novou sekvencí o délce obvykle 9–11 AA (Borrow et al., 2019). Veškeré mutace na C-konci NPM1 vedou ke ztrátě tryptofanových zbytků v pozicích 288 a 290, případně pouze ke ztrátě Trp290, které jsou důležitou součástí sekvence NoLS (Falini et al., 2006b, 2005) a jsou nezbytné pro správné prostorové uspořádání CTD (Grummitt et al., 2008; Scognamiglio et al., 2016). Chybně sbalená CTD NPM1 není schopna efektivně vázat NA a zajistit tak jadérkovou lokalizaci proteinu (Bañuelos et al., 2013; Chiarella et al., 2013; Grummitt et al., 2008). Zároveň je v mutNPM1 vytvořen nový NES, jenž interakcí s exportním proteinem CRM1 významně zvyšuje účinnost transportu aberantního proteinu do cytoplazmy (Castagnola, 2005; Falini et al., 2006b). V závislosti na typu mutace vznikají různé motivy NES lišící se afinitou k CRM1. Částečné rozrušení NoLS, způsobené delecí pouze Trp290, je spojeno s účinnějšími NES, zatímco delece obou tryptofanů bývá asociována se slabšími signály NES (Arregi et al., 2015; Bolli et al., 2007). Specifickým případem je tzv. super-NES vytvořený v důsledku inzerce v exonu 5 či 6 genu *NPM1*, který exportuje mutovaný protein do cytoplazmy za přítomnosti neporušeného NoLS (Martelli et al., 2021; Martelli, 2018). Imunohistochemický průkaz cytoplazmatického NPM1 je tedy jedna z rychlých a spolehlivých metod identifikujících přítomnost mutace NPM1 (Falini et al., 2006c). Typ mutace je následně zjišťován sekvenací exonu 12 nebo, v případě nekorelujících nálezů, sekvenací celého *NPM1* genu (Falini et al., 2021; Martelli et al., 2021). NPM1 významně přispívá k udržování genomové stability a hraje zásadní roli během embryogeneze, což dokládá skutečnost, že se mutace *NPM1* vyskytují pouze v heterozygotním stavu (Grisendi et al., 2005). Výzkum na myších modelech prokázal, že homozygotní mutace *NPM1* je letální již v embryonálním stadiu (Grisendi et al., 2005). Přibližně u 80 % dospělých pacientů s AML s mutNPM1 se objevuje duplikace TCTG (c.860_863dupTCTG), označovaná jako mutace A. K dalším rozšířenějším typům mutací NPM1 patří typy B (c.863_864insCATG) a D (c.863_864insCCTG), jejichž frekvence výskytu v součtu společně s typem A dosahuje 90 % (Borrow et al., 2019; Döhner et al., 2005; Schnittger et al., 2005; Thiede et al., 2006).



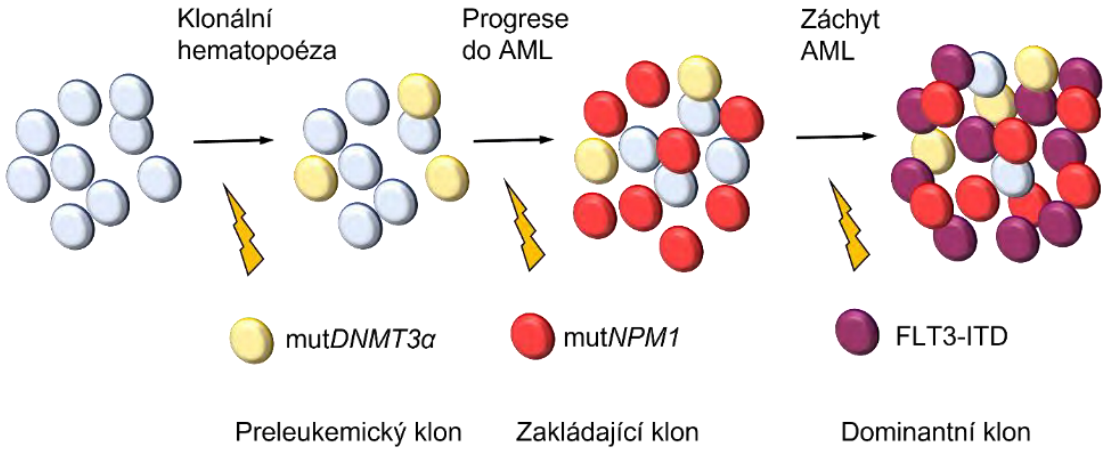
Obrázek 4: Schéma kódující sekvence NPM1 a nejčastější typy mutací u AML s mutNPM1. Transkripční varianta NPM1 (NPM1.1) je složená z 11 exonů, naprostá většina mutací postihuje exon 12 (mezi nukleotidy 863 a 864). Nejrozšířenější typy mutací – A, B a D – jsou způsobeny inzercí (v případě mutace typu A duplikací) 4 bp (červeně), následkem čehož dochází ke ztrátě Trp288 a Trp290 (modře), klíčových AA fungujících jako NoLS. MutNPM1 nově získává sekvenci NES (zeleně) zprostředkovávající účinný export proteinu do cytoplazmy. NPM1 = nukleofosmin 1; AML s mutNPM1 = akutní myeloidní leukémie s mutovaným nukleofosminem 1; AA = aminokyselina; NoLS = jadérkový lokalizační signal; NES = jaderný exportní signál. (Upraveno podle (Hindley et al., 2021))**.**

Existuje více než 100 různých typů mutací genu *NPM1* (Ahmad et al., 2009; Bacher et al., 2014; Borrow et al., 2019; Cazzaniga et al., 2005; Döhner et al., 2005; Duployez et al., 2018; Falini et al., 2006c, 2005; Ivey et al., 2016; Jeon et al., 2013; Jeziskova et al., 2017; Kawaguchi-Ihara et al., 2016; Pianta et al., 2009; Rau and Brown, 2009; Schnittger et al., 2005; Suzuki, 2005; Thiede et al., 2006; Venanzi et al., 2021; Verhaak et al., 2005), přičemž se jen zřídka vyskytují v jiném exonu než 12 – identifikovány byly např. v exonech 5 (Martelli et al., 2021; Martelli, 2018), 6 (Martelli et al., 2016), 9 (Mariano et al., 2006) a 11 (Albiero et al., 2007; Falini et al., 2021; Martelli et al., 2016; Pianta et al., 2009; Pitiot et al., 2007). Navzdory vysoké variabilitě detekovaných mutací indukují všechny vyjma jeden případ (Pianta et al., 2009) přesun mutNPM1 do cytoplazmy, což se jeví jako klíčová událost v procesu leukemogeneze (Bolli et al., 2007; Brunetti et al., 2017; Falini et al., 2009; Mariano et al., 2006; Martelli et al., 2021; Venanzi et al., 2021). Kromě cytoplazmatické lokalizace mutNPM1 mají pacienti s detekovanou mutací v genu *NPM1* většinou NK (Falini et al., 2005), často také zvýšený počet leukocytů (De Propris et al., 2011; Suzuki, 2005) a miskovitý (tzv. cup-like) tvar jádra blastů (Chen et al., 2009; Kroschinsky et al., 2008). Charakteristická je dále nízká exprese lidských leukocytárních antigenů (HLA; human leukocyte antigens) HLA-DR, absence povrchových znaků CD34 a CD133 (Falini et al., 2005) a naopak přítomnost CD33 (De Propris et al., 2011;

Nomdedeu et al., 2011). S výskytem NPM1 mutací také pozitivně koreluje přítomnost interních tandemových duplikací (ITD; internal tandem duplication) v genu *FLT3* (z angl. *FMS-like tyrosine kinase-3*) (Cancer Genome Atlas Research Network et al., 2013; Falini et al., 2005; Suzuki, 2005) nebo mutace v genu pro DNA metyltransferázu 3 alfa (DNMT3α; DNA methyltransferase 3 alpha) (Cancer Genome Atlas Research Network et al., 2013; Thol et al., 2011). Naopak další mutace typické pro AML, např. v genech pro RUNX1 (z angl. *runt-related transcription factor 1*) (Gaidzik et al., 2011; Mendler et al., 2012; Susanne Schnittger et al., 2011; Tang et al., 2009) a p53 (Haferlach et al., 2008; Kadia et al., 2016; Suzuki, 2005) nebo částečné tandemové duplikace genu MLL (z angl. *mixed lineage leukemia*) (Sun et al., 2017), bývají u pacientů s NPM1 mutací detekovány jen zřídka. Mutace NPM1 bez přítomnosti FLT3-ITD a/nebo bez mutace DNMT3α je u pacientů s NK pozitivním prognostickým ukazatelem příznivé odpovědi na indukční terapii (Bezerra et al., 2020; Döhner et al., 2005; Falini et al., 2005; Gale et al., 2015; Guryanova et al., 2016; Heiblig et al., 2021; Herold et al., 2020; Kuželová et al., 2021a; Schnittger et al., 2005; Thiede et al., 2006). Nicméně názor na prognostický význam mutace v genu *DNMT3α* u AML s mutNPM1 není zcela jednotný, zřejmě ovlivňuje spíše pravděpodobnost relapsu než celkové přežití (OS; overall survival) (Gaidzik et al., 2013; Oñate et al., 2022). Chromozomové abnormality vyskytující se asi u 15 % AML s mutNPM1 postihují nejčastěji chromozomy 8, 4, Y, 9 a 21 a obvykle nejsou považovány za prognosticky nepříznivý nález (Haferlach et al., 2009). Incidence NPM1 mutací u dětí je ve srovnání s dospělou populací nízká (přibližně 8 % versus 30 %) a také se mezi těmito skupinami liší četnosti výskytů jednotlivých typů mutace (Brown et al., 2007; Cazzaniga et al., 2005; Hollink et al., 2009; Mullighan et al., 2007; Rau and Brown, 2009; Thiede et al., 2007). Vzhledem k charakteristickým klinickým, morfologickým, imunofenotypovým, cytogenetickým a molekulárně genetickým rysům onemocnění byla AML s mutNPM1 klasifikována Světovou zdravotnickou organizací (WHO; World health organization) jako samostatná kategorie (Arber et al., 2016). Mutace je rekurentní, což znamená, že ačkoliv v remisi dojde u většiny pacientů ke snížení výskytu patologického klonu nesoucího mutaci NPM1 pod detekovatelnou mez, v době relapsu tento klon znovu expanduje. Hladina transkriptu mutNPM1 či detekce mutace *NPM1* na úrovni DNA je proto vhodným markerem pro sledování průběhu onemocnění a minimální zbytkové choroby (MRD; minimal residual disease) (Dvorakova et al., 2010; Ivey et al., 2016; Jain et al., 2014; Schnittger et al., 2009).

2.6.1.1 Klonální vývoj u AML s mutNPM1 a prognóza onemocnění

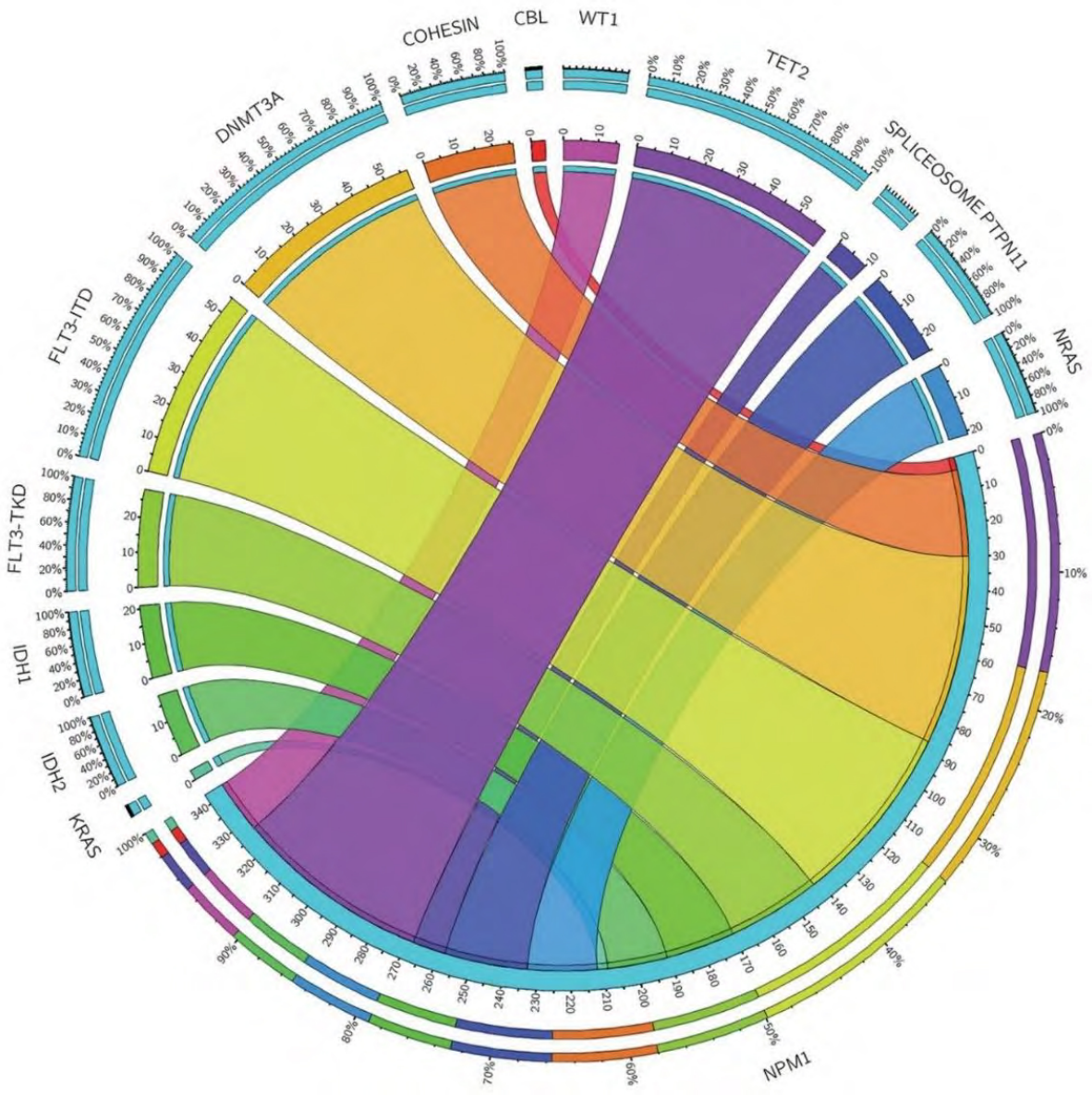
Patogeneze AML představuje vícestupňový proces. V době diagnózy je pro AML typická heterogenita klonů nesoucích jak mutace preleukemické, umožňující klonální expanzi, tak mutace získané v pozdějším období a způsobující propuknutí samotné nemoci (Grimwade et al., 2016; Morita et al., 2020; Welch et al., 2012) (**Obr. 5**). Preleukemické klony mohou unikat léčbě a skrytě přetrvávat v remisi i v relapsu, zatímco skladba klonů nesoucích mutace spojené s leukemogenezí se v průběhu onemocnění mění (Corces-Zimmerman et al., 2014; Shlush et al., 2014).



Obrázek 5: Klonální heterogenita AML. Preleukemické mutace (např. v genu *DNMT3α*), vedou k expanzi postižených buněk. S výskytem dalších mutací (např. v genu *NPM1*), se nemaligní klonální hematopoéza přetváří na maligní. V době stanovení diagnózy AML bývá zachycena variabilita buněčné populace, přičemž populace zdravých buněk bývá postupně utlačena leukemickým klonem. AML = akutní myelodní leukémie; mut*DNMT3α* = mutovaný gen *DNA metyltransferáza 3 alfa*; mut*NPM1* = mutovaný gen *nukleofosmin 1*; FLT3-ITD = interní tandemová duplikace genu *FLT3* (z angl. *FMS-like tyrosine kinase-3*). (Upraveno podle (Grimwade et al., 2016)).

NPM1 mutace se málokdy vyskytuje samostatně (Mer et al., 2021) (**Obr. 6**), častěji bývá asociována s přítomností dalších mutací, velmi často v genech regulujících metylaci DNA (*DNMT3α*, *TET2* (z angl. *ten-eleven translocation methylcytosine dioxygenase 2*), isocitrát dehydrogenáza 1/2 (IDH1/2; isocitrate dehydrogenase 1/2)) (Bezerra et al., 2020; Cancer Genome Atlas Research Network et al., 2013; Papaemmanuil et al., 2016; Patel et al., 2012), sestřih RNA (*SRSF2* (z angl. *serine and arginine rich splicing factor 2*), *SF3B1* (z angl. *splicing factor 3b subunit 1*)) (Patel et al., 2017; Pettersson et al., 2021) a buněčnou signalizaci (*FLT3*, *NRAS* (z angl. *neuroblastoma RAS viral oncogene homolog*) či *PTPN11* (z angl. *protein tyrosine phosphatase non-receptor type 11*)) (Bezerra et al., 2020; Cancer Genome Atlas Research Network et al., 2013; Papaemmanuil et al., 2016; Patel et al., 2012) nebo kódujících

kohezinové proteiny (*RAD21*, *SMC1A* (z angl. *structural maintenance of chromosomes 1A*), *SMC3* (z angl. *structural maintenance of chromosomes 3*), *STAG1* (z angl. *stromal antigen 1*), *STAG2* (z angl. *stromal antigen 2*)) (Patel et al., 2017; Thol et al., 2014).



Obrázek 6: Grafické znázornění četností výskytu mutací u AML s mutNPM1 a jejich vzájemných vztahů. Mutace NPM1 bývá nejčastěji asociována s mutacemi ve znázorněných genech. Číslo uvnitř kruhového grafu udává počet případů s výskytem dané mutace v kohortě 120 pacientů s mutNPM1. Číslo vně grafu udává procentuální zastoupení uvedených ko-mutací u pacientů s konkrétní mutací. AML = akutní myeloidní leukémie; NPM1 = nukleofosmin 1; KRAS = z angl. *Kirsten rat sarcoma virus*; IDH = isocitrát dehydrogenáza; FLT3-TKD = mutace v tyrozinkinázové doméně genu *FLT3* (z angl. *FMS-like tyrosine kinase-3*); FLT3-ITD = interní tandemová duplikace genu *FLT3*; DNMT3A = DNA metyltransferáza 3 alfa; COHESIN = kohezin; CBL = z angl. *Casitas B-lineage lymphoma*; WT1 = z angl. *Wilms' tumor 1*; TET2 = z angl. *ten-eleven translocation methylcytosine dioxygenase 2*; PTPN11 = z angl. *protein tyrosine phosphatase non-receptor type 11*; NRAS = z angl. *neuroblastoma RAS viral oncogene homolog*. (Převzato z (Patel et al., 2017)).

Současný výskyt změn v genech *NPM1*, *DNMT3α* a *FLT3* je jednou z nejčastěji prokázaných kombinací genotypů u AML (Bezerra et al., 2020; Cancer Genome Atlas Research Network et al., 2013; Papaemmanuil et al., 2016; Patel et al., 2017; Potter et al., 2019). Přestože mutace NPM1 pravděpodobně představuje spouštěcí faktor ve vývoji AML, objevuje se patrně jako sekundární událost, které předchází vznik tzv. zakladatelských (founder) mutací, a to obvykle v genech *DNMT3α*, *IDH1*, *IDH2* či *TET2* (Papaemmanuil et al., 2016; Patel et al., 2017). Rozlišení zakladatelských a pozdních mutací vychází z posuzování výskytu jednotlivých mutací ve vztahu k variantní alelické frekvenci mutací (VAF; variant allele frequency). U mutací v genech *DNMT3α*, *TET2, IDH1/2*, *RAD21*, *SMC1A*, *SMC3*, *SRSF2* a *SF3B1* byl detekován medián VAF > 39 %, zatímco u mutace v genu *NPM1* byla popsána hodnota mediánu VAF okolo 17 % (Patel et al., 2017). Mutace v genech *FLT3*, *NRAS* či *PTPN11* byly identifikovány s mediánem VAF srovnatelným či nižším než mutace *NPM1*, což naznačuje, že tyto mutace nejsou primární genetickou událostí (Patel et al., 2017). Tento koncept postupné akumulace mutací, kdy se mutace *NPM1* objevuje až jako subklonální defekt, je dále podpořen popsanými perzistencemi mutací v genech *DNMT3α*, *IDH1*, *IDH2* u některých pacientů s AML s mutNPM1 v kompletní remisi (Corces-Zimmerman et al., 2014) nebo souborem pacientů s AML s mutNPM1 relabujících jako wtNPM1, nesoucích však jiné mutace stanovené v době diagnózy (Höllein et al., 2018; Suzuki, 2005). U pacientů s AML s mutNPM1, kteří dosáhli remise, může být však rozvoj AML či MDS bez původní mutace *NPM1* důsledkem předchozí protinádorové léčby AML, tzv. therapy-related AML, kdy se AML vyvíjí ze sekundárního leukemického klonu vzniklého, případně vyselektovaného, v průběhu předchozí léčby (Cocciardi et al., 2019; Herold et al., 2017; Krönke et al., 2013).

Přítomnost dalších specifických mutací pak výrazně ovlivňuje prognózu pacientů s AML s mutNPM1. Negativní klinický dopad mají zejména mutace FLT3-ITD či mutace DNMT3α (Alpermann et al., 2016; Döhner et al., 2005; Metzeler et al., 2016; Papaemmanuil et al., 2016; Renneville et al., 2012; Verhaak et al., 2005). V případě FLT3-ITD pravděpodobně záleží na hodnotě alelického poměru (AR; allelic ratio) mutované a nemutované alely (Pratcorona et al., 2013; S. Schnittger et al., 2011). Pacienti s mutNPM1, FLT3-ITD s AR < 0,5 ($FLT3\text{-}ITD^{low}$) a s NK spadají podle aktuální WHO klasifikace do kategorie AML s příznivým rizikem (Döhner et al., 2017). Nicméně byl zaznamenán nepříznivý vývoj onemocnění, pokud tito pacienti nepodstoupili alogenní transplantaci krvetvorných kmenových buněk (allo-HSCT; allogeneic hematopoietic stem cell transplantation) v první remisi (Sakaguchi et al., 2018). Zajímavý

pohled na úlohu FLT3-ITD v patogenezi AML s mutNPM1 nabízí nová studie Mer *et al.*, která na základě analýzy expresního profilu rozlišuje dva molekulární podtypy AML s mutNPM1. Podskupina označovaná jako „primitive“ obsahovala značnou frakci kmenových buněk, v podskupině charakterizované jako „committed“ byly buňky diferencovanější (Mer et al., 2021). Analýza obou skupin z hlediska přítomnosti FLT3-ITD ukázala, že přibližně u třetiny pacientů expresní profil neodpovídal stavu této mutace. Zároveň zhruba u 40 % pacientů exprimujících wtFLT3, bez ohledu na uvedené podskupiny, došlo k relapsu, což není u pacientů s tímto genotypem obvyklé. Vyvstává tedy otázka, zda indikace těchto pacientů k allo-HSCT v první remisi nezvýší jejich šanci na přežití. U primitivního typu AML s mutNPM1 byly dále prokázány horší výsledky z hlediska OS, avšak vyšší citlivost k léčbě vybranými kinázovými inhibitory bez ohledu na přítomnost FLT3-ITD.

Některé klinické studie uvádějí, že faktory jako OS, stav kompletní remise, přežívání bez relapsu (RFS; relaps free survival) či bez sledované události (EFS; event free survival) u pacientů s AML s mutNPM1 závisejí také na typu přítomné mutace *NPM1*. Data publikace Koh *et al.* signalizují, že u FLT3-ITD negativních pacientů s mutací typu A lze očekávat příznivější prognózu než s mutací typu B a Q6 (Koh et al., 2009). Autoři Alpermann *et al.* uvádějí, že výskyt mutace typu A u FLT3-ITD pozitivních pacientů a mutace typu A a D u DNMT3α pozitivních pacientů bývá asociován s horší prognózou ve srovnání s výskytem jiných typů mutací *NPM1* u těchto skupin pacientů (Alpermann et al., 2016). Jiná analýza ukazuje zkrácení RFS a EFS ve skupině AML pacientů s mutací typu A oproti skupině s jinými mutacemi *NPM1*, a to bez ohledu na přítomnost FLT3-ITD (Selim et al., 2016). Trend k prodloužení OS u pacientů s mutacemi *NPM1* jiného typu než A, kteří obdrželi v rámci terapie hypometylační látky, zaznamenává nedávná studie Sciumè *et al.* (Sciumè et al., 2019). Také byla nalezena spojitost mezi dosažením MRD negativity a typem mutace *NPM1* u intenzivně léčených AML pacientů (Heiblig et al., 2019). V této studii nositelé mutace typu A dříve dosáhli MRD negativity, ale delší OS byl zaznamenán naopak u skupiny nemocných s jinými typy mutací *NPM1* než s typem A. Na druhou stranu některé studie neshledaly žádnou souvislost mezi typem mutace *NPM1* a průběhem onemocnění pacientů s AML s mutNPM1 a NK (Park et al., 2012; Pastore et al., 2014). Podobně konfliktní data vyplývají ze studií potenciálního prognostického významu hodnoty VAF pro mutovanou alelu *NPM1* (Abbas et al., 2019; Linch et al., 2020; Patel et al., 2018). Kromě molekulárního profilu patří mezi další faktory ovlivňující prognózu AML s mutNPM1 zejména věk (Becker et al., 2010; Juliusson et al., 2020; Lachowiez et al., 2020;

Suzuki, 2005), karyotyp (Angenendt et al., 2019), počet leukocytů (de Jonge et al., 2011) a diskutované je i etnikum (Chauhan et al., 2013; Koh et al., 2009).

2.6.1.2 Role mutace NPM1 v leukemogenezi

Proteinový produkt mutovaného genu *NPM1* získává nové vlastnosti, které mají dopad na jeho biologickou aktivitu. Změna vnitrobuněčného rozložení představuje nové pole působnosti pro mutNPM1 v cytoplazmě a zároveň snížená hladina wtNPM1 v jadérku vlivem exprese z jediné alely a také v důsledku jeho oligomerizace s mutNPM1 vede k nedostatečné funkci proteinu. Zda k progresi maligní transformace aktivně přispívají nové funkce mutNPM1 či spíše ztráta funkce wtNPM1, je stále předmětem diskuse (Grisendi et al., 2006; Heath et al., 2017). Nedávno publikovaná data demonstrují, že fenotyp leukemické populace – nadměrná proliferace a stupeň buněčné diferenciace – závisí na přítomnosti mutNPM1 v cytoplazmě: buňky s mutNPM1 mají podstatně vyšší expresi *HOX* genů, které jsou zásadní pro vývoj kmenových buněk, zatímco v normálních diferencovaných buňkách bývá jejich hladina nízká (Brunetti et al., 2018). Cytoplazmatická akumulace NPM1, ať už v důsledku mutace v jeho CTD nebo patologické fúze genů, se tedy jeví jako klíčový faktor leukemogeneze (Bolli et al., 2007; Brunetti et al., 2017; Falini et al., 2009; Martelli et al., 2021; Venanzi et al., 2021). Konkrétní mechanismus účinku mutNPM1 v patogenezi AML však stále není zcela objasněn. Výsledky řady studií dokládají, že v důsledku mutace NPM1 dochází k narušení genomové stability, ztrátě funkcí tumor supresorů p14Arf a p53, deregulaci apoptózy a změnám v expresi C-MYC a dalších proteinů.

2.6.1.2.1 Genomová nestabilita

NPM1 jako jeden ze substrátů komplexu cyklin-dependentní kináza 2/cyklin E asociuje s centrozomem během mitózy a brání svojí přítomností jeho duplikaci. Na konci G1 fáze stimuluje specifická fosforylace v pozici Thr199 NPM1 jeho uvolnění z centrozomu, čímž je zahájen proces duplikace centrozomu (Okuda, 2002; Okuda et al., 2000; Tokuyama et al., 2001). Při přechodu buňky do anafáze pak dochází k opětovné defosforylaci Thr199 a asociaci NPM1 s centrozomem. Ačkoli bylo pozorováno, že myši haploinsuficientní pro *NPM1* vykazují zvýšené riziko rozvoje maligní transformace, aberantní počet centrozomů a strukturní či početní chromozomální abnormality (Grisendi et al., 2005; Sportoletti et al., 2008), skutečnost, že pacienti s NPM1 mutací mají převážně NK (Falini et al., 2005; Schlenk et al., 2008) signalizuje, že tato mutace zřejmě nemá zásadní vliv na udržení stability genomu na úrovni chromozomů.

2.6.1.2.2 Deregulace tumor supresorových proteinů p14Arf a p53

V buňkách nesoucích mutNPM1 je snížená hladina wtNPM1, který má potenciál regulovat stabilitu p53. Lze tedy předpokládat, že částečné vyřazení genu *NPM1* přispěje skrze deregulaci p53 k maligní transformaci buněk. Experimenty na myších embryích a embryonálních fibroblastech s vyřazenou činností genu *NPM1* však odhalily vyšší hladinu a zachovanou aktivitu p53 navozující předčasnou buněčnou senescenci a zastavení buněčného cyklu (Colombo et al., 2005; Grisendi et al., 2005). Úloha wtNPM1 při regulaci aktivity p53 se tedy nejeví jako klíčová pro přeměnu zdravých buněk na buňky nádorové. Protože C-koncové mutace NPM1 neruší interakci mezi proteiny NPM1 a p53, je komplex p53-mutNPM1 transportován do cytoplazmy (Holoubek et al., 2021). Ačkoli p53 uvolněný do cytoplazmy může indukovat apoptózu (Comel et al., 2014), aberantní lokalizace tohoto proteinu může narušit regulaci buněčného cyklu a podpořit proliferaci a přežívání buněk, což představuje zvýšené riziko maligní transformace (Senapedis et al., 2014). Biologická funkce p53 tedy může být v buňkách s mutNPM1 narušena změnou jeho vnitrobuněčné lokalizace.

Jak bylo zmíněno výše, interakce NPM1 s p14Arf zprostředkovává jadérkovou lokalizaci p14Arf a chrání jej před degradací (Colombo et al., 2005; Korgaonkar et al., 2005). V důsledku mutace NPM1 však dochází k přesunu komplexu mutNPM1-p14Arf do cytoplazmy a k výraznému snížení poločasu rozpadu p14Arf. Buňky s redukovanou hladinou p14Arf nejsou schopny adekvátní buněčné odpovědi na stres – utlumení proliferace a zastavení buněčného cyklu skrze aktivaci p53 (Besten et al., 2005; Colombo et al., 2006). Není však jasné, zda tento mechanismus skutečně urychluje nádorovou transformaci buňky nesoucí mutNPM1 a vede tak k rozvoji leukémie (Besten et al., 2005). Mimoto je v buňkách exprimujících mutNPM1 snížena schopnost p14Arf indukovat sumoylaci proteinů MDM2 a wtNPM1, a to i přesto, že je frakce wtNPM1, stejně jako p14Arf, vlivem interakce s mutNPM1 přesunuta do cytoplazmy, čili se oba proteiny teoreticky mohou dostat do těsné blízkosti (Besten et al., 2005). Tyto experimenty byly ovšem prováděny na myší linii NIH-3T3 a pro využití výsledků v humánní medicíně je proto potřeba ověřit je též na lidských buňkách, ať už buněčných liniích nebo primárních buňkách AML pacientů.

2.6.1.2.3 Porucha regulace apoptózy

Mutace NPM1 v jeho C-koncové doméně má vliv na jeho regulační funkci v procesu apoptózy. MutNPM1 je v cytoplazmě schopen interagovat s aktivními formami kaspázy-6 (CASP-6; caspase-6) a kaspázy-8 (CASP-8; caspase-8), přičemž s CASP-6 asociuje jeho

C-koncová oblast (186–259 AA), zatímco s CASP-8 jeho N-koncová doména. MutNPM1 vazbou k těmto kaspázám potlačuje jejich proteázové aktivity hrající zásadní roli v programované buněčné smrti a také diferenciační aktivity v myeloidních buňkách (Leong et al., 2010). Takový zásah do kaspázové kaskády může přispívat ke zvýšenému přežití leukemických buněk a k zastavení terminální diferenciace blastů.

NPM1 je zodpovědný za jaderný import produktu genu *GADD45α* (Gao et al., 2005), regulátoru kontrolního bodu přechodu mezi fázemi G2 a M (Wang et al., 1999). Podnětem k zesílené expresi GADD45α je určitý typ poškození DNA (Fornace et al., 1989). Jeho zvýšenou frakci lze pak detekovat jak v cytoplazmě, tak v jádře, kam je vzhledem k absenci vlastního NLS dopravován v komplexu s NPM1 (Gao et al., 2005). Cytoplazmaticky lokalizovaný NPM1 pravděpodobně není schopen zajistit translokaci GADD45α do jádra, v důsledku čehož tento protein nemůže uplatnit inhibiční účinek na průběh buněčného cyklu.

2.6.1.2.4 Vysoká hladina proteinu C-MYC

Jako transkripční faktor reguluje C-MYC genovou expresi asi 10–15 % lidských genů (Zeller et al., 2006). Translokace, amplifikace nebo mutace protoonkogenu *MYC* patří k častým nálezům v mnoha typech nádorů (Adhikary and Eilers, 2005; Bahram et al., 2000; Beroukhim et al., 2010; Dalla-Favera et al., 1982; Taub et al., 1982). Jeho hladina v buňce je mimo jiné regulována vazbou NPM1 na Fbw7γ (z angl. *F-box/WD repeat domain-containing 7*). Fbw7γ patří mezi E3-ubiquitin ligázy zajišťující specifickou ubiquitinylaci a degradaci onkoproteinu C-MYC. Pro správnou funkci Fbw7γ je nezbytná jeho jadérková lokalizace a stabilizace zprostředkovaná interakcí s NPM1. V důsledku mutace NPM1, která nezamezuje vazbě proteinů mutNPM1 a Fbw7γ, však dochází také k cytoplazmatické delokalizaci Fbw7γ a následně pak k jeho degradaci. V buňkách nesoucích mutNPM1 tak nemůže probíhat účinné odbourávání proteinu C-MYC (Bonetti et al., 2008). Imunohistochemické stanovení onkoproteinu C-MYC v kostní dřeni AML pacientů prokázalo, že vyšší míra exprese C-MYC pozitivně koreluje s nálezem mutace NPM1 (Ohanian et al., 2019)

2.6.1.2.5 Změny v expresi *HOX* genů

Homeotické geny spadají do velké skupiny regulačních genů s velmi konzervovanou strukturou DNA a hrají klíčovou roli v organogenezi během raného embryonálního vývoje (Gehring, 1987; Graham et al., 1989; Lewis, 1978; Mark et al., 1997) a také v procesu hematopoézy (Lawrence et al., 1996). Deregulace *HOX* genů byla popsána u různých nádorů včetně leukémií (Blatt et al., 1988; Celetti et al., 1993; Grier et al., 2005; Nakamura et al.,

1996). U AML s mutNPM1 dochází k výrazně zvýšené expresi genů *HOXA* a *HOXB*, která vede k expanzi hematopoetických progenitorů a ke zvýšené schopnosti jejich sebeobnovy (Alcalay et al., 2005; Dovey et al., 2017; Spencer et al., 2015; Tregnago et al., 2021; Vassiliou et al., 2011). Nedávná studie Brunetti *et al.* prokázala, že zvýšená exprese *HOX* genů není jen projevem vlastností leukemických buněk charakteristických pro kmenové buňky, nýbrž má přímou souvislost s výskytem mutace *NPM1* a s lokalizací mutNPM1 v cytoplazmě. Odstranění mutNPM1 z cytoplazmy navodilo nižší expresi *HOX* genů následovanou diferenciací buněk AML (Brunetti et al., 2018).

2.6.1.3 Cílená léčba AML s mutNPM1

Léčba AML s kurativním záměrem byla až donedávna omezena převážně na užívání chemických sloučenin s cytotoxickými účinky. Standardní indukční režim vychází z protokolu „3+7“ publikovaného v roce 1973 (Yates et al., 1973), kdy se podává 7 dní cytarabin (ARA-C; arabinosylcytosine) a z toho první 3 dny společně s antracykliny (Lichtman, 2013). Tento protokol je však spojen s řadou nepříznivých účinků a není vhodný pro všechny pacienty. Zejména starší pacienti, pro které by tato intenzívní léčba přinášela více rizik, jsou proto léčeni šetrnějšími protokoly zahrnujícími použití hypometylačních analogů cytidinu (azacytidin nebo decitabin) nebo nižší dávky ARA-C. V posledních letech však dochází k rychlému vývoji molekulárně genetických vyšetřovacích metod, což umožňuje efektivnější hledání a identifikaci nových prediktivních markerů AML a s tím související rozvoj nové generace léků cíleně zasahujících klíčové mechanismy leukemogeneze (Lai et al., 2019).

AML s mutNPM1 za současné absence jiných mutací dobře odpovídá na indukční chemoterapii. Nedostatečná funkce NPM1 v důsledku jeho mutace vyvolává tzv. ribozomální stres, který se projeví inhibicí MDM2 a tedy stabilizací p53, což zvyšuje náchylnost takové buňky k apoptóze (Derenzini et al., 2018). Zvýšená citlivost AML buněk s mutNPM1 k protinádorové léčbě může být způsobena také nižší aktivitou NF-κB v důsledku jeho interakce s mutNPM1 (Cilloni et al., 2008; Zhang et al., 2016) a podpořena účinnou T-buněčnou imunitní odezvou namířenou proti mutNPM1 (Forghieri et al., 2019; Greiner et al., 2013). Nicméně vedle kardiotoxicity antracyklinů, která představuje jednu z hlavních limitací léčby, přetrvává zejména u starších pacientů riziko relapsu či rezistence na standardní chemoterapii (Ossenkoppele et al., 2016; Roboz, 2012). A tak možnosti cílené terapie přinášejí novou naději na dlouhodobé přežití nemocných. Doposud nebylo objasněno, zda při vzniku AML s mutNPM1 a při její léčbě hraje důležitější roli nedostatečná funkce wtNPM1 či změna

lokalizace mutNPM1 a jeho interakčních partnerů vyvolaná jeho aberantní lokalizací. Existuje několik léčebných strategií zamezujících asociaci mutované formy NPM1 s jeho přirozenou formou a s jeho vazebnými partnery a/nebo ovlivňujících lokalizaci a stabilitu NPM1.

Jedním z možných přístupů je inhibice oligomerizace NPM1. Tento účinek byl identifikován u malých molekul YTR107 (5-((N-benzyl-1H-indol-3-yl)-methylene)pyrimidine-2,4,6(1H,3H,5H) trione) (Penthala et al., 2015; Sekhar et al., 2014, 2011; Traver et al., 2021) a NSC348884 (di-[((6-methyl-1H-benzo[d]imidazol-2-yl)methyl)((5-methyl-3-oxo-3H-indol-2-yl)methyl)]) aminoethane) (Qi et al., 2008) vázajících se k NTD NPM1. Experimentální léčivo NSC348884 bylo posuzováno několika studiemi popisujícími nejen jeho efekt na oligomerní komplexy NPM1, ale celou škálu rozmanitých účinků, od utlumení buněčné proliferace, přes aktivaci p53 a indukci apoptózy, po snížení adhezivity buněk (Balusu et al., 2011; Phi et al., 2019; Qi et al., 2008; Šašinková et al., 2021). Dále byl na nádorových buněčných liniích odvozených z adenokarcinomu prostaty a z lymfomu plášťových buněk a na leukemických buňkách popsán synergický efekt NSC348884 s působením doxorubicinu (DOX; doxorubicin) (Qi et al., 2008) a s působením kyseliny all-trans-retinové (ATRA; all-trans-retinoic acid) a ARA-C (Balusu et al., 2011). Nicméně klíčová schopnost NSC348884 blokovat tvorbu oligomerů NPM1 byla v nedávné studii zpochybněna (Šašinková et al., 2021). Podrobněji se touto problematikou zabývám v publikaci NSC348884 cytotoxicity is not mediated by inhibition of nucleophosmin oligomerization zahrnuté v kapitole Výsledky. Alternativní způsob, jakým dosáhnout rozpadu NPM1 oligomerů, představuje použití synteticky vytvořených oligonukleotidů RNA, tzv. RNA aptamerů (Jian et al., 2009). Jako nejvhodnější z navržených variant byl vyhodnocen aptamer 1A1 vykazující afinitu pro NPM1 v jeho centrální oblasti (114–186 AA). Vazba aptameru 1A1 k NPM1 navodila disociaci jeho oligomerů, s čímž by zřejmě mohl souviset přesun NPM1 z jadérka do nukleoplazmy, který byl pozorován v buněčných liniích exprimujících 1A1. Společně s NPM1 byl do nukleoplazmy uvolněn také p14Arf stabilizující p53, což je ve shodě se zaznamenanou vyšší expresí p53 a rozsáhlejší apoptózou v těchto buňkách (Jian et al., 2009).

Druhou strategií cílené léčby AML s mutNPM1 je inhibice interakce mezi NPM1 a jinými proteiny. Do této kategorie léčivých přípravků spadá syntetický peptid NucAnt 6L (N6L) původně cílený na receptorový NCL na povrchu nádorových buněk vykazující však afinitu také pro NPM1 (Destouches et al., 2011). Vazba N6L k NTD NPM1 brání její účasti v proteinových interakcích, což představovalo slibný předpoklad zvýšené apoptózy buněk nesoucích

mutNPM1 v důsledku uvolnění proteinů regulujících apoptózu z cytoplazmatických komplexů s mutNPM1. Tato domněnka se však nepotvrdila, a naopak bylo dokumentováno, že N6L preferenčně spouští apoptózu v buňkách obsahujících wtNPM1 (De Cola et al., 2018). Nicméně ošetření buněk kombinací N6L s DOX a/nebo ARA-C vedlo k vzestupu toxicity těchto cytostatik u leukemických linií OCI-AML2, exprimující pouze wtNPM1, i OCI-AML3, exprimující mutNPM1, což přináší slibnou perspektivu pro terapii AML (De Cola et al., 2018), přestože její mechanismus pravděpodobně neovlivňuje NPM1. Nověji vyvíjenou skupinou bioaktivních molekul jsou inhibitory interakce mezi APE1 a NPM1 (Poletto et al., 2016). Stejně jako NPM1, také APE1 bývá v nádorových buňkách nadměrně exprimována nebo aberantně lokalizována, přičemž právě zvýšená exprese obou proteinů patří k faktorům souvisejícím s vyšší agresivitou nádoru (Chen et al., 2018; Yuan et al., 2017). Vyšší hladina NPM1 asociovaného s APE1 tedy může přispívat ke zvýšené buněčné proliferaci, invazivitě a rezistenci nádorových buněk vůči terapii. U molekul blokujících interakci APE1-NPM1 byla pozorována schopnost tlumit proliferaci nádorových buněk a zvyšovat jejich citlivost vůči látkám poškozujícím DNA (Poletto et al., 2016).

Alternativní léčebná strategie spočívá v regulaci PTM NPM1. Je známo, že deregulace aktivity proteinových kináz hraje významnou roli v rozvoji nádorového bujení, což z těchto proteinů činí atraktivní cíl protinádorové léčby (Giamas et al., 2007). Jednu z významných serin-threoninových kináz, jejíž změny v aktivitě a expresi byly popsány u řady solidních a hematologických nádorů, představuje kaseinová kináza 2 (CK2; casein kinase 2) (Chua et al., 2017). NPM1 patří mezi dobře prostudované substráty CK2 (Szebeni et al., 2003) a zároveň byl identifikován jako klíčový ligand rozpoznávaný specifickým proapoptotickým peptidem CIGB-300 (dříve pod názvem P15-Tat) (Perera et al., 2009). Tento inhibitor cílí na fosfoakceptorovou oblast substrátu CK2, kterou je v případě NPM1 Ser125, čímž se toto místo stává pro proteinkinázu nepřístupné (Perea et al., 2018, 2004). Zamezení fosforylace NPM1 v důsledku navázání CIGB-300 zřejmě narušuje stabilitu jadérka, což následně vede k masivnímu vyvolání apoptózy (Perera et al., 2009).

Další možná strategie je založena na tzv. hypotéze vyhladovělého jadérka vycházející z předpokladu, že wt frakce NPM1 v jadérku AML buněk s mutNPM1 je nezbytná pro jejich přežití, a že tedy odstranění zbytkového NPM1 z jadérka indukuje jadérkový stres, který vyústí v apoptózu leukemických buněk (Falini et al., 2011; Federici and Falini, 2013). Mezi látky této skupiny uvolňující NPM1 z jadérka je řazen např. TMPyP4 (tetra-N-methyl-4-pyridyl porphyrin)

vázající G-kvadruplexy rDNA s vyšší afinitou než NPM1 (Chiarella et al., 2013). Paradoxně ale byly rozsáhlejší toxické účinky TMPyP4 zaznamenány u buněčné linie nesoucí wtNPM1 (Chiarella et al., 2013), zatímco životaschopnost a rozsah apoptózy AML buněk s mutNPM1 nebyly tímto inhibitorem příliš ovlivněny (De Cola et al., 2014). Ačkoli tedy tato léčba měla najít uplatnění v terapii AML s mutNPM1, může být nakonec využita spíše v nádorových tkáních nadměrně exprimujících NPM1. Skutečnost, že v AML buňkách s mutNPM1 je v důsledku snížené hladiny wtNPM1 v jadérku určitá míra jadérkového stresu, se však odráží ve zvýšení jejich citlivosti vůči určitým chemoterapeutickým látkám (Falini et al., 2011). Např. ActD (Falini et al., 2015; Gionfriddo et al., 2021; B. Y. M. Yung et al., 1985), daunorubicin (Chan and Chan, 1999), DOX (Chan et al., 1987) nebo platinová cytostatika cisplatina a oxaliplatina (Burger et al., 2010) prohlubují jadérkový stres akumulací NPM1 v nukleoplazmě, čímž dochází k porušení integrity jadérka a tyto buňky podstupují apoptózu (Rubbi, 2003).

Jiná léčebná strategie využívá selektivní degradace mutNPM1 při současném zachování hladiny wtNPM1. Jako slibný kandidát pro tento způsob léčby byl navržený oxid arsenitý, ATRA a jejich kombinace (El Hajj et al., 2015; Martelli et al., 2015) a analog imunomodulátoru imiquimodu EAPB0503 (1-(3-methoxyphenyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine) (Nabbouh et al., 2017). Tato léčiva vyvolávají proteazomální degradaci mutNPM1, obnovují jadérkovou/jadernou lokalizaci wtNPM1 a v závislosti na koncentraci, době působení a použité buněčné linii s různou účinností zastavují buněčný růst a spouštějí apoptózu. Z aktivních přírodních látek byl na buněčných liniích nesoucích mutNPM1 testován flavonoid deguelin (Yi et al., 2015) a katechin epigallokatechin-3-gallát (Chi et al., 2014). Obě tyto rostlinné složky způsobovaly pokles hladiny mutNPM1, potlačovaly buněčnou proliferaci a indukovaly buněčnou smrt.

Horší prognóza AML je mimo jiné asociována s nadměrnou expresí receptoru CRM1 (Kojima et al., 2013), který zprostředkovává jaderný export řady proteinů včetně NPM1, nejznámějších tumor supresorů p53, p21 či retinoblastomového proteinu, a onkoproteinů jako je např. produkt kódovaný abnormálním genem vzniklým fúzí *BCR* (z angl. *breakpoint cluster region protein*) a *ABL1* (z angl. *Abelson tyrosine-protein kinase 1*) (Turner et al., 2012). Inhibice CRM1 tzv. selektivními inhibitory jaderného exportu (SINE; selective inhibitor of nuclear export) se tedy jeví jako slibná léčebná modalita v terapii nádorových onemocnění (Azizian and Li, 2020). Nejlépe prostudovaným SINE je selinexor (KPT-330), jehož efekt na relokalizaci mutNPM1 do jádra byl již zdokumentován (Gu et al., 2018). Zatímco u solidních

nádorů byla jeho účinnost jen omezená, pro terapii hematologických malignit zahrnujících AML, non-Hodgkinův lymfom a mnohočetný myelom (pro jehož léčbu byl nedávno schválen americkým Úřadem pro kontrolu potravin a léčiv (Research, 2019)), se jeví selinexor velice perspektivní (Azizian and Li, 2020). Monoterapie selinexorem u nemocných s refrakterní/relabující AML (Garzon et al., 2017) stejně jako přidání selinexoru ke standardní indukční (ARA-C + daunorubicin) a konsolidační (ARA-C) chemoterapii u pacientů s nově diagnostikovanou AML s vysokým rizikem (Pardee et al., 2020; Sweet et al., 2016) byly shledány jako účinné a bezpečné. Jak již bylo dříve zmíněno, NPM1 interaguje s regulátorem myeloidní diferenciace PU.1, přičemž tato interakce není narušena C-koncovou mutací NPM1. To má za následek přesun komplexu mutNPM1-PU.1 do cytoplazmy, kde PU.1 nemůže stimulovat diferenciaci granulocytů a monocytů. Použití selinexoru blokujícího jaderný export mutNPM1-PU.1 nastartovalo v AML buňkách s mutNPM1 monocytickou diferenciační dráhu (Gu et al., 2018). Podobný efekt by se dal očekávat i u dalších proteinů, jejichž lokalizace je ovlivněna interakcí s mutNPM1. Podrobné studium účinku selinexoru na lokalizaci p53, který je prokazatelně delokalizován do cytoplazmy vlivem interakce s mutNPM1, ale ukázalo, že redistribuce obou proteinů po ošetření selinexorem probíhá nezávisle. V tomto případě je nutné vzít v úvahu rovněž přímý vliv selinexoru na p53, přičemž výsledný mechanismus působení v buňkách s mutNPM1 je pravděpodobně souhrou několika různých signálních kaskád (Holoubek et al., 2021).

Poměrně nově uvažovaným léčebným postupem je využití farmakologického chaperonu, tedy malé molekuly zprostředkující sbalení proteinu (Convertino et al., 2016). Látka s takovýmto účinkem v případě mutNPM1 indukuje sbalení CTD do formy charakteristické pro wtNPM1, což vytváří předpoklad pro jeho relokalizaci zpět do jadérka (Chiarella et al., 2013; Falini et al., 2006b; Urbaneja et al., 2017), kde mutNPM1 ztrácí antiapoptotickou funkci asociovanou se svým cytoplazmatickým výskytem. V této kategorii léčiv byl zvažován avrainvillamid rozpoznávající C-koncovou doménu NPM1 (Wulff et al., 2007). Jedná se o alkaloid s antiproliferativní aktivitou, který v leukemických buňkách stimuluje přesun mutNPM1 z cytoplazmy do jadérka a nukleoplazmy a navozuje apoptózu (Andresen et al., 2016; Mukherjee et al., 2015), nicméně schopnost regulace prostorového uspořádání CTD mutNPM1 u něj zatím nebyla prokázána. Dalším terapeutikem s antiproliferativními a proapoptotickými účinky na buněčnou linii OCI-AML3, které též

indukuje přesun mutNPM1 z cytoplazmy do jádra, je diterpen oridonin, ani u něj však přesný mechanismus působení na mutNPM1 není známý (Li et al., 2014).

Relokalizace mutNPM1 do jadérka bylo docíleno také použitím některých činidel narušujících syntézu DNA. Na pacientských buňkách nesoucích mutNPM1 a na buněčné linii OCI-AML3 bylo prokázáno, že po jejich ošetření etoposidem či ARA-C dochází k přesunu cytoplazmatického mutNPM1 do jadérka (Bailey et al., 2019).

Jak bylo uvedeno v kapitole 2.6.1.2.5 Změny v expresi *HOX* genů, výskyt mutace NPM1 koreluje se zvýšenou expresí *HOX* genů (Alcalay et al., 2005; Spencer et al., 2015), kterou v buňkách s mutNPM1 regulují metyltransferázy MLL a DOT1L (z angl. *disruptor of telomeric silencing 1-like*) (Kühn et al., 2016). V řízení exprese genů *HOX* a zajištění obnovy krevních buněčných komponent hraje důležitou roli vazba meninu (MEN; menin) k MLL (Chen et al., 2006; Novotny et al., 2009). Kombinace epigenetických léčiv inhibujících interakci MEN-MLL a enzym DOT1L aktivovala diferenciaci a utlumila proliferaci leukemických blastů s mutNPM1 za současného snížení exprese genů *HOX* (Kühn et al., 2016). V buňkách ošetřených těmito léčivy nebyla zaznamenána změna v celkové metylaci konkrétních histonů, úroveň metylace ve specifických lokusech souvisejících s expresí *HOX* genů však byla významně snížena. Epigenetické regulátory tedy představují další slibnou kategorii protinádorových léčivých přípravků pro terapii AML s mutNPM1.

Současným trendem na poli léčby AML je cílená terapie kombinovaná s dalšími chemoterapeutickými režimy. Antiapoptotický protein B-buněčných lymfomů BCL-2 (z angl. *B-cell lymphoma-2*) je považován za atraktivní terapeutický cíl pro svoji schopnost podporovat přežívání blastů a vysokou expresi v těchto buňkách, která je asociována s horší prognózou onemocnění (Campos et al., 1993). V léčbě starších pacientů s AML s mutNPM1 dosahuje velmi dobrých výsledků kombinovaná léčba hypometylačních látek s Venetoclaxem, inhibitorem BCL-2 (DiNardo et al., 2019; Lachowiez et al., 2020). Tato kombinovaná terapie byla shledána účinnou také u pacientů se sekundární AML či s cytogenetickým nálezem předznamenávajícím špatnou prognózu (DiNardo et al., 2019).

Velmi aktuálním přístupem k léčbě AML s mutNPM1 je imunoterapie využívající NPM1 jako potenciální cíl pro aktivované T-lymfocyty (TL; T lymphocytes). Bylo nalezeno několik imunogenních peptidů z mutované části mutNPM1 (neoantigenů), zároveň ale byly specifikovány i sekvence schopné vyvolat imunitní odezvu a pocházející z nemutované části proteinu. V mutované oblasti NPM1 byly charakterizovány dva nonapeptidy (NPM1#1 a

NPM1#3) specifické pro alely HLA-A2, které byly schopny stimulovat $CD8^+$ TL. Aktivace $CD8^+$ TL byla monitorována pomocí sekrece granzymu B a interferonu γ. Prodloužené peptidy derivované z jednoho z těchto dvou neoantigenů (NPM1#1) aktivovaly také $CD4^+$ TL, které by tedy mohly ve spolupráci s $CD8^+$ TL cíleně eliminovat leukemické buňky (Greiner et al., 2012). Analýza souboru 25 pacientů s AML s mutNPM1 následně ukázala asociaci mezi prodloužením OS a specifickou imunitní reakcí $CD8^+$ TL rozpoznávajících peptidové fragmenty NPM1#1 a NPM1#3 (Greiner et al., 2013). V nedávné studii byl identifikován antigenně specifický receptor na povrchu T-lymfocytů (TCR; T-cell receptor) rozpoznávající další konkrétní peptid z mutované C-koncové oblasti NPM1 v komplexu s HLA-A2. Tento TCR byl pomocí retroviru vložen do $CD4^+$ a $CD8^+$ TL zdravých jedinců, nosičů alely HLA-A2. Reaktivita takto upravených TL byla testována na buněčných liniích OCI-AML2 a OCI-AML3 a také na buňkách pacientů s AML s mutací a bez mutace NPM1. Účinnost těchto modifikovaných TL byla prokázána jak u leukemických buněk nesoucích mutNPM1, tak na myším modelu s mutNPM1 (van der Lee et al., 2019). Zdokumentována byla také efektivita a specifita modifikovaných T-lymfocytů nesoucích chimérický antigenní receptor rozpoznávající mutovaný peptid z mutNPM1 prezentovaný HLA-A2 (Xie et al., 2021). V důsledku heterooligomerizace a cytoplazmatické lokalizace mutNPM1 je pro štěpení na peptidové fragmenty lépe přístupný také wtNPM1, případně interakční partneři NPM1. Tím lze získat rozsáhlejší soubor antigenních peptidů, které mohou být prezentovány na molekulách hlavního histokompatibilního komplexu. Studie zaměřující se na zjištění frekvence výskytu jednotlivých HLA alel I. třídy v souboru pacientů s AML s mutNPM1 uvádí, že určité HLA alely (A*02, B*07, B*18, B*40, C*07) se u pacientů s mutNPM1 vyskytují s nižší frekvencí než je běžné ve zdravé populaci či u AML pacientů, kteří nenesou mutNPM1. Peptidy predikované pro asociaci s těmito alelami pocházejí převážně z nemutované sekvence NPM1. Navíc byla u pacientů s AML s mutNPM1 prokázána souvislost mezi výskytem těchto méně zastoupených alel a vývojem nemoci a pravděpodobností dlouhodobého přežití (Kuželová et al., 2018, 2015). Nádorové tkáně často vykazují silnou expresi ligandu programované buněčné smrti 1 (PD-L1; programmed cell death ligand 1) (Dong et al., 2002) rozpoznávajícího membránový receptor - protein programované buněčné smrti 1 (PD-1; programmed cell death protein 1) exprimovaný především na aktivovaných T- a B-lymfocytech a některých myeloidních buňkách imunitního systému (Freeman et al., 2000). V posledních letech je interakci PD-L1-PD-1 v oblasti imunoterapie věnována stále větší pozornost. Ve skupině 30 pacientů s AML (polovina s mutací NPM1) byla u jedinců nesoucích

mutNPM1 pozorována vyšší exprese PD-L1, a to zejména v leukemických kmenových buňkách (LSC; leukemia stem cells). Nicméně v expresi PD-L1 na úrovni mediátorové RNA (mRNA; messenger RNA) nebyly mezi LSC a zbylou frakcí buněk zjištěny signifikantní rozdíly (Greiner et al., 2017). Korelace mezi hladinami mRNA a proteinu PD-L1 nebyla potvrzena ani ve studii Brodska *et al.*, byla však nalezena asociace mezi množstvím PD-L1 exprimovaného na povrchu buněk a poměrem dvou jeho transkripčních variant (Brodská et al., 2016b). V nedávné době bylo navíc prokázáno, že NPM1 reguluje expresi PD-L1 pomocí interakce mezi NPM1 a promotorem PD-L1 (Qin 2020). Imunoterapie je proto slibným směrem pro léčbu AML s mutNPM1, ať už jako monoterapie nebo jako podpůrný prostředek pro jiné způsoby léčby.

3 CÍLE

Tématem dizertační práce jsou mutace v genu *NPM1* a jejich dopad na funkci proteinu. Přirozená forma NPM1 se vyskytuje převážně ve formě pentamerů a je lokalizována v buněčném jadérku. Charakteristická mutace v CTD, která je velmi často detekována u pacientů s AML, má za následek přesun mutovaného proteinu do cytoplazmy. NTD NPM1 zajišťuje oligomerizaci a interakci NPM1 s mnoha dalšími proteiny a její sekvence zůstává v mutNPM1 neporušena. Změna vnitrobuněčné lokalizace mutNPM1 proto ovlivňuje nejen jeho biologickou funkci, ale také lokalizaci jeho interakčních partnerů a wtNPM1. Případné terapeutické zásahy do oligomerizační domény NPM1 s cílem rozrušit komplexy obsahující mutNPM1 by mohly obnovit jadérkovou lokalizaci a aktivitu wtNPM1 i správnou funkci jeho interakčních partnerů. Cílem práce bylo zavést mutace do genu *NPM1* a vyšetřit jejich funkční následky, zejména popsat vliv klinicky relevantních změn v C-koncové části proteinu na vybrané interakce a pomocí manipulací s N-koncovou doménou charakterizovat roli oligomerizace v signalizaci NPM1. Dále popsat vliv vybraných léčiv na tvorbu oligomerů a na interakční potenciál wtNPM1 a mutNPM1.

Práce má tyto dílčí cíle:

1) V návaznosti na výsledky diplomové práce porovnávající expresi a lokalizaci mutNPM1 v buněčných liniích HeLa a HEK-293T prokázat pomocí imunoprecipitace (IP; immunoprecipitation) konstruktů značených zesíleným zeleným fluorescenčním proteinem (eGFP; enhanced green fluorescent protein) interakci mezi exogenní a endogenní formou NPM1.
2) Zavést a optimalizovat metody pro detekci oligomerů NPM1 *in vitro* pomocí nativní polyakrylamidové gelové elektroforézy (nat PAGE; native polyacrylamide gel electrophoresis) a seminativní polyakrylamidové gelové elektroforézy (semi-nat PAGE; semi-native polyacrylamide gel electrophoresis), IP a *in vivo* prostřednictvím konfokální mikroskopie a časově rozlišené fluorescenční mikroskopie (FLIM; fluorescence lifetime imaging microscopy) v kombinaci s fluorescenčním rezonančním přenosem energie (FRET; fluorescence resonance energy transfer).
3) Analyzovat vliv mutace v CTD NPM1 na jeho oligomerizaci a interakci se známými interakčními partnery. Pro bližší zkoumání byl zvolen jadérkový fosfoprotein NCL, jehož

interakce s wtNPM1 byla dříve popsána a jeho funkce je v mnoha procesech komplementární s funkcí NPM1.

4) Sledování účinku vybraných cytotoxických léčiv na interakci NPM1-NCL, lokalizaci NPM1 a na stabilitu jeho oligomerů.
5) S využitím molekulárního klonování připravit mutované varianty NPM1 s narušenou oligomerizační doménou, které představují modelový systém pro experimenty studující oligomerizaci NPM1, a charakterizovat jejich vlastnosti pomocí *in vitro* i *in vivo* metod.
6) Prostřednictvím zavedených metod analyzovat vliv malé molekuly NSC348884 deklarované jako inhibitor oligomerizace NPM1 na tvorbu oligomerů a na interakční potenciál wtNPM1 a jeho mutované formy asociované s AML.

4 VÝSLEDKY

Výsledková část je souhrnem čtyř publikací, které prošly řádným recenzním řízením a byly uveřejněny v impaktovaných vědeckých časopisech. Výsledky popsané v těchto publikacích jsme získali v naší laboratoři v době mého doktorského studia a můj podíl na nich je specifikován na konci kapitol věnovaných jednotlivým publikacím.

4.1 Publikace č. 1: Localization of AML-related nucleophosmin mutant depends on its subtype and is highly affected by its interaction with wild-type NPM1

Shrnutí obsahu: V této práci detailně charakterizujeme vnitrobuněčnou lokalizaci exogenního fluorescenčně značeného NPM1 v závislosti na typu C-koncové mutace a hladině endogenního NPM1. Pro studium lokalizace NPM1 jsme využívali tři expresní systémy, HEK-293T, Hela a NIH-3T3, lišící se mírou exprese vnášených konstruktů a relativní hladinou endogenního NPM1. Zatímco buněčná linie HEK-293T je charakteristická vysokou expresí vnášeného plazmidu, buňky NIH-3T3 vykazují standardní amplifikaci plazmidové DNA i endogenního NPM1. U buněčné linie HeLa je přítomno více kopií genu *NPM1* (Macville et al., 1999), a proto se tyto buňky vyznačují vysokou hladinou endogenního NPM1. Mimoto jsme zavedli postup detekce cytoplazmatického mutNPM1 (bez ohledu na typ mutace) v blastech pacientů s mutNPM1 metodou imunofluorescenčního značení fixovaných buněk.

Je známo, že schopnost oligomerizace NPM1 není narušena C-koncovou mutací, což umožňuje tvorbu heterooligomerů mezi přirozenou a mutovanou formou NPM1 (Bolli et al., 2009). Dosud identifikované typy mutací NPM1 jsou charakterizovány ztrátou jednoho nebo obou tryptofanů (Trp290, případně i Trp288) ze sekvence zajišťující lokalizaci NPM1 v jadérku a nahrazením této oblasti sekvencí zprostředkující transport NPM1 z jádra. Zkonstruovali jsme proto plazmidy nesoucí dvě skupiny NPM1 mutací. V první skupině byla nejčastější NPM1 mutace typu A (80 % případů) a jí podobné typy B a Nm, které postrádají oba tryptofany, ale jejichž získaný NES má relativně nízkou afinitu k exportnímu proteinu CRM1. Druhá skupina plazmidů obsahovala typy E a H se zachovaným Trp288 a s NES s vysokou afinitou k CRM1 (Bolli et al., 2007). Vnitrobuněčnou lokalizaci všech výše zmíněných mutovaných forem jsme sledovali pomocí fluorescenčního mikroskopu v buněčné linii HEK-293T. MutNPM1 typu A, B a Nm vykazovaly téměř identickou, převážně cytoplazmatickou lokalizaci, zatímco mutNPM1 typu E a H byly přítomny v jadérku i cytoplazmě současně. Protože se v rámci jedné skupiny chovaly dané varianty srovnatelně, prezentovali jsme v publikaci z každého souboru nejčastější typy mutací, tedy A a B z prvního a E z druhého souboru. V liniích NIH-3T3 a HeLa jsme pozorovali výrazný nárůst podílu buněk s mutovanou formou lokalizovanou v cytoplazmě a jadérku zároveň. Exprese mutNPM1 typu A na úrovni proteinu byla u buněčných linií HEK-293T, NIH-3T3 a HeLa stanovena pomocí imunoblotů. Při porovnání snímků z fluorescenčního mikroskopu a imunoblotů jsme našli korelaci mezi počtem buněk s výhradně cytoplazmatickou lokalizací mutNPM1 a poměrem exprese exogenního NPM1 vůči

endogennímu NPM1 v buněčném lyzátu. To nás vedlo k hypotéze, že heterooligomery vznikají též mezi exogenní a endogenní formou NPM1 a že vyšší podíl mutNPM1 lokalizovaného v jadérku je důsledkem vyšší exprese endogenního NPM1. Tato domněnka byla následně potvrzena metodou IP využívající systém agarózových kuliček s navázanými peptidy s vysokou afinitou k eGFP nebo k monomernímu červenému fluorescenčnímu proteinu 1 (mRFP1; monomeric red fluorescent protein 1) – GFP/RFP-Trap. Pomocí GFP-Trapu jsme precipitovali eGFP-značené varianty wtNPM1 (eGFP_wtNPM1) a mutNPM1 (eGFP_mutNPM1) typu A a typu E z buněčných lyzátů HEK-293T a HeLa. U obou buněčných linií se endogenní NPM1 koprecipitoval spolu s exogenní, přirozenou i mutovanou, formou NPM1, přičemž přítomnost endogenního NPM1 byla vyšší pro precipitaci přirozenou formou NPM1. Zároveň jsme prokázali vyšší poměr endogenního NPM1 v precipitátech linie HeLa, což souhlasí s předpokladem, že zvýšená exprese endogenního NPM1 má za následek větší podíl jeho molekul v heterooligomerech.

Tato publikace vychází z dat naměřených v rámci řešení mé diplomové práce. Je však významně rozšířena o (1) imunofluorescenční detekci variant NPM1 na vzorcích od pacientů s AML, kterým byla vzhledem k vysokému výskytu leukocytů (převážně blastů) v periferní krvi provedena leukodeplece (Fig. 1); (2) veškeré statistické analýzy týkající se vnitrobuněčné lokalizace mutNPM1 (Fig. 2b, 3b, 3c, 4c); (3) kvantitativní stanovení hladiny exogenního NPM1 (wt a s mutacemi typu A, B a E) vůči endogennímu NPM1 pomocí techniky imunoblotu a hodnocení účinnosti transfekce jednotlivých konstruktů u buněčné linie HEK-293T (Fig. 2c); (4) imunobloty srovnávající různou hladinu endogenního NPM1 a rozdílnou expresi exogenního mutNPM1 typu A u buněčných linií HEK-293T, NIH-3T3 a HeLa (Fig. 4b, 4d); (5) imunoprecipitační experimenty prokazující interakci mezi endogenní a exogenní formou NPM1 (Fig. 5).

Můj podíl na práci: (1) Příprava mutovaných forem NPM1 technikou molekulárního klonování a jejich transfekce do buněčných linií HEK-293T, HeLa a NIH-3T3; (2) provedení lokalizačních experimentů využívajících konfokální mikroskopii (live-cell imaging, imunofluorescence); (3) příprava buněčných lyzátů z adherentních buněčných linií HEK-293T, NIH-3T3 a HeLa, provedení PAGE v přítomnosti dodecylsíranu sodného (SDS; sodium dodecyl sulfate), IP pomocí GFP-Trapu a vyhodnocení western blotů (WB; western blot); (4) příprava obrázků a účast na psaní manuskriptu.

4.2 Publikace č. 2: Monitoring of nucleophosmin oligomerization in live cells

Shrnutí obsahu: Biologická aktivita a oligomerizace NPM1 spolu úzce souvisejí (Hingorani et al., 2000; Mitrea et al., 2014; Prinos et al., 2011). Jednotlivé molekuly NPM1 spolu v oligomerech interagují skrze NTD (Hingorani et al., 2000), která zároveň zprostředkovává řadu interakcí s dalšími proteiny, např. p14Arf (Bertwistle et al., 2004; Itahana et al., 2003), APE1 (Vascotto et al., 2009), MEF/ELF4 (z angl. *E74-like factor 4*) (Ando et al., 2013). Zásah do oligomerizační domény by tedy mohl vést ke změnám v interakční kapacitě NPM1. U proteinu, který je díky interakci s mutovanou formou NPM1 delokalizován do cytoplazmy, se předpokládá, že porušením interakční domény může dojít k jeho uvolnění z komplexu s mutNPM1 v cytoplazmě a jeho přesun zpět do jádra by pak mohl způsobit obnovení jeho biologické funkce. NTD NPM1 tudíž představuje zajímavý terapeutický cíl (Balusu et al., 2011; Jian et al., 2009; Qi et al., 2008). Dle dostupné literatury byla oligomerizace NPM1 studována přednostně metodami *in vitro* (Chan and Chan, 1995; Duan-Porter et al., 2014; Enomoto et al., 2006; Hingorani et al., 2000; Lin et al., 2016; Liu and Yung, 1999; Mitrea et al., 2014; Prinos et al., 2011; Yung and Chan, 1987). Rozhodli jsme se k analýze NPM1 oligomerizace *in vitro* experimenty doplnit *in vivo* přístupy založenými na fluorescenčních proteinech. Fluorescenční proteiny jsme tedy pomocí technik molekulárního klonování připojili ke studovaným proteinům, což nám umožnilo pro sledování NPM1 oligomerizace zavést a validovat dvě *in vivo* metody využívající fluorescenční mikroskopii – kros-korelační analýzu dvou fluorescenčních signálů detekovaných ve spektrálně odlišených kanálech ((ccN&B; cross-correlation number and brightness assay) kombinovanou s pulzně prokládaným buzením (PIE; pulsed interleaved excitation)), což jsou metody fluorescenční korelační spektroskopie, a metodu časově rozlišené fluorescenční spektroskopie FLIM-FRET. Kroskorelace dvou spektrálně odlišených signálů udává statistickou míru podobnosti časového průběhu signálů, které přísluší dvěma odlišně barevným fluoreskujícím značkám. Hodnota tohoto parametru zrcadlí společný pohyb různě barevných molekul vyskytujících se v molekulárních komplexech ve sledovaném vzorku. Jako značky jsme použili fluorescenční proteiny eGFP a mRFP1. Tyto značky mohou být zároveň použity i pro sledování vzájemné vzdálenosti označených molekul prostřednictvím FRET. Technika FLIM-FRET mapuje dobu dohasínání fluorescence zeleně fluoreskujícího proteinu eGFP, který sloužil jako donor, z čehož může být následně usuzováno na vzdálenost mezi označenými molekulami čili potenciální interakci. Jako akceptor jsme použili červený fluorescenční protein mRFP1. Obě fluorescenční

metody použité pro *in vivo* sledování oligomerizace NPM1 vyžadují vhodné kontroly. Jako pozitivní kontrola pro PIE-ccN&B metodu byl použit wtNPM1 značený současně oběma fluorescenčními proteiny: mRFP1 na N-konci a eGFP na C-konci. Tento model ukazoval nejvyšší míru korelace zeleného a červeného signálu. Negativní kontrolu představovala směs dvou typů proteinů, eGFP_wtNPM1 a volného mRFP1. V měření FLIM-FRET jsme přítomnost FRET ověřili metodou založenou na fotodestrukci (vybělení) akceptoru. Po vybělení akceptoru by mělo v případě přítomnosti přenosu energie dojít k prodloužení doby dohasínání a nárůstu intenzity fluorescence donoru a k návratu ke stavu bez FRET. FRET jsme tímto způsobem pozorovali ve směsi proteinů eGFP_wtNPM1 a wtNPM1 značeného mRFP1 (mRFP1_wtNPM1). To je v souladu s předpokladem, že molekuly NPM1 spolu interagují v oligomerech (Schmidt-Zachmann et al., 1987; Umekawa et al., 1993; Yung and Chan, 1987). Negativní kontrolu v analýze FLIM-FRET představovala dvojice NCL značeného eGFP a NCL značeného mRFP1. NCL vykazuje převážně jadérkovou lokalizaci srovnatelnou v transfekovaných buňkách s NPM1 (Schmidt-Zachmann and Nigg, 1993) a zároveň nedochází k výraznému zkrácení doby dohasínání fluorescence u směsi barevně značených molekul NCL ve srovnání s dobou dohasínání fluorescence pozorovanou pro eGFP-značený NCL bez přítomnosti červené formy, čímž bylo ověřeno, že k podstatnému zkrácení doby dohasínání eGFP v případě eGFP_wtNPM1-mRFP1_wtNPM1 nedochází v důsledku nespecifické interakce molekul vyskytujících se v jadérku ve vysokých koncentracích a tudíž velmi blízko u sebe. S takto nastaveným systémem metod vhodných pro sledování interakcí *in vivo* jsme plánovali naměřit data pro molekuly NPM1 s poškozenou oligomerizací. Jedním z inhibitorů oligomerizace NPM1 má být léčivo NSC348884 (Balusu et al., 2011; Qi et al., 2008). V našich experimentech však tato látka v doporučených koncentracích indukovala buněčnou apoptózu a snížení buněčné adhezivity, což nám ztížilo studium jejího efektu metodami založenými na fluorescenční mikroskopii. Podrobněji jsme se jejímu vlivu věnovali v pozdější publikaci (Šašinková et al., 2021). K ovlivnění oligomerizace NPM1, a tedy k validaci *in vivo* metod, jsme dále použili metody molekulárního klonování a provedli inhibující zásahy do oligomerizační domény popsané v literatuře. Cys21 je uveden jako klíčová AA pro tvorbu oligomerů NPM1 (Huang et al., 2013; Prinos et al., 2011). Jeho substituce za Phe či Trp inhibuje dle studie *Prinos et al.* tvorbu pentamerů NPM1 (Prinos et al., 2011), substituce za Ala podle *Huang et al.* pak tento proces částečně narušuje (Huang et al., 2013). V jiné publikaci se dokládá, že jadérková lokalizace variant NPM1 přímo souvisí s jejich výskytem v oligomerních komplexech (Enomoto

et al., 2006; Jian et al., 2009). Pokud by tedy následkem inhibující mutace v Cys21 došlo k poškození oligomerizace NPM1, měla by být narušena lokalizace této formy v jadérku a takový protein by se měl ve zvýšené koncentraci vyskytovat v nukleoplazmě. Za využití konfokální mikroskopie jsme tedy porovnali vnitrobuněčnou lokalizaci fluorescenčně značených wtNPM1, NPM1 mutantů p.Cys21Ala a p.Cys21Phe v buňkách HEK-293T. Shledali jsme, že všechny tři formy NPM1 vykazují srovnatelnou lokalizaci, s převahou signálu z jadérek a minoritní nukleoplazmatickou frakcí. Toto pozorování naznačuje, že mutace v Cys21 buď neinhibují oligomerizaci NPM1, nebo rozpad NPM1 komplexů v jadérku nevede k relokalizaci NPM1 do nukleoplazmy. Následně jsme ověřovali vliv substituce Cys21 na oligomerizaci NPM1 metodou FLIM-FRET. Grafy ukazující rozdělení dob dohasínání fluorescence eGFP v jednotlivých obrazových elementech v závislosti na poměru mezi intenzitou v červeném a zeleném detekčním kanálu pro červeně a zeleně značené dvojice wtNPM1, NPM1 p.Cys21Ala a p.Cys21Phe vykazovaly pro všechny dvojice stejný trend, a to snížení doby dohasínání fluorescence eGFP s rostoucím poměrem intenzit akceptoru ku donoru. Tento výsledek svědčí o přítomnosti oligomerů u všech testovaných variant NPM1. Dále u těchto variant byla provedena analýza PIE-ccN&B. Koeficienty kroskorelace testovaných dvojic červeně a zeleně značeného wtNPM1, p.Cys21Ala, p.Cys21Phe byly vzájemně statisticky neodlišitelné, což rovněž podpořilo naši domněnku o schopnosti oligomerizace Cys21-mutovaných NPM1 molekul. Závěrem jsme uvedené *in vivo* metody doplnili o imunoprecipitační analýzu, pro kterou byly použity lyzáty HEK-293T buněk transfekovaných vždy jednou zeleně značenou variantou wtNPM1, p.Cys21Ala a p.Cys21Phe. Endogenní NPM1 se koprecipitoval ve srovnatelné míře jak s wtNPM1, tak s formami NPM1 mutovanými v Cys21, což doložilo zachovanou vazebnou schopnost těchto mutantů k wtNPM1. Lze tedy shrnout, že pomocí dynamických metod fluorescenční spektroskopie je možné sledovat oligomery tvořené molekulami NPM1 a že substituce Cys21 neruší schopnost NPM1 vytvářet oligomery.

Můj podíl na práci: (1) Příprava NPM1 mutantů p.Cys21Ala a p.Cys21Phe technikou molekulárního klonování a jejich transfekce do buněčné linie HEK-293T; (2) provedení lokalizačních experimentů využívajících konfokální mikroskopii (live-cell imaging); (3) příprava buněčných lyzátů z adherentní buněčné linie HEK-293T, provedení SDS-PAGE, IP pomocí GFP-Trapu a vyhodnocení WB.

4.3 Publikace č. 3: AML-associated mutation of nucleophosmin compromises its interaction with nucleolin

Shrnutí obsahu: Tato studie popisuje vliv C-koncové mutace NPM1 na jeho oligomerizační vlastnosti a jeho interakci s významným jadérkovým fosfoproteinem NCL. Prostřednictvím nat PAGE a semi-nat PAGE jsme prokázali, že stabilita oligomerů tvořených mutNPM1 je nižší než stabilita wtNPM1 komplexů, což je důležitý poznatek pro vývoj léčiv cílených na oligomerizaci NPM1. Přestože mnohé proteiny interagují s NPM1 prostřednictvím jeho NTD zodpovědné za oligomerizaci NPM1, některé, jako např. p53 (Colombo et al., 2002; Lambert and Buckle, 2006) či NCL (Li et al., 1996), interagují s NPM1 skrze jeho střední a C-koncovou oblast. NCL je multifunkční fosfoprotein nacházející se převážně v jadérku a spolupracující s NPM1 především při ribogenezi, opravách DNA a buněčné proliferaci (Scott and Oeffinger, 2016; Tajrishi et al., 2011). Naše výsledky získané pomocí IP a konfokální mikroskopie dokládají, že interakce NPM1 a NCL je podstatně narušena přítomností C-koncové mutace NPM1. V případě NPM1 mutace typu A, která vede ke ztrátě NoLS, jde o úplnou absenci interakce mutNPM1-NCL, zatímco pro mutaci typu E, která způsobuje ztrátu pouze jednoho ze dvou tryptofanů podstatných pro jadérkovou lokalizaci, zůstává tato interakce zčásti zachována. NCL interaguje s oblastí NPM1 nezasaženou mutací (187–245 AA) (Li et al., 1996), sekvence vazebného místa pro NCL zůstává proto v mutNPM1 nezměněna. Narušení interakce NPM1-NCL v přítomnosti mutace může být zapříčiněno konformační změnou NPM1 nebo fyzickou separací interakčních partnerů odrážející jejich odlišnou vnitrobuněčnou lokalizaci. Druhou uvedenou možnost jsme testovali za využití cytotoxických léčiv ActD nebo Leptomycinu B (LmB; Leptomycin B), která způsobují redistribuci NPM1 a NCL do nukleoplazmy. ActD, známé antibiotikum inhibující syntézu RNA (Goldberg and Rabinowitz, 1962; Reich et al., 1961), indukuje kondenzaci jadérek a nukleoplazmatickou relokalizaci jadérkových proteinů (Brodská et al., 2016a; Kalousek et al., 2005). LmB inhibuje jaderný exportní protein CRM1 (Kudo et al., 1998), což vede k akumulaci jeho klientních proteinů, k nimž patří i NPM1, v jádře (Bolli et al., 2007; Falini et al., 2006b). Kolokalizace fluorescenčně značených exogenních proteinů NCL a mutNPM1 navozená účinkem ActD nebo LmB byla v buňkách HEK-293T ověřena fluorescenční mikroskopií, přítomnost proteinového komplexu byla studována metodou IP. Zatímco interakce wtNPM1-NCL zůstala v buňkách ošetřených cytostatiky neporušená, interakce mezi mutNPM1 a NCL detekována nebyla. Lze tedy předpokládat, že neschopnost mutNPM1 vázat NCL není způsobena jejich odlišnou

vnitrobuněčnou lokalizací. Přítomnost mutNPM1 v buňce má za následek relokalizaci části wtNPM1 do cytoplazmy (Bolli et al., 2009; Brodská et al., 2017) a tedy snížení hladiny NPM1 dostupného pro tvorbu komplexu NPM1-NCL. Za předpokladu neměnné exprese NCL dochází v těchto buňkách k vychýlení poměru relativních koncentrací wtNPM1 a NCL, což může mít významný dopad na buněčné procesy regulované těmito proteiny. Porovnali jsme tedy hladinu exprese NCL v blastech pacientů s mutNPM1 a bez mutNPM1 a v buněčných liniích OCI-AML3 (exprimující mutNPM1) a OCI-AML2 (exprimující wtNPM1). Z výsledků je patrné, že úroveň exprese NCL není závislá na přítomnosti mutNPM1, což může mít vliv na kooperaci těchto jadérkových proteinů.

Další léčivo, které jsme se rozhodli blíže charakterizovat ve vztahu k NPM1, byla ATRA. ATRA představuje významného zástupce v terapii akutní promyelocytární leukémie (APL; acute promyelocytic leukemia) (Burnett et al., 2015; Castaigne et al., 1990; Fenaux et al., 1997; Huang et al., 1988; Lo-Coco et al., 2013), jejíž vznik je u většiny nemocných s APL spjat s balancovanou translokací t(15;17)(q24;q21) zahrnující nádorový supresor promyelocytický leukemický protein (PML; promyelocytic leukemia protein) a receptor RARα (Alcalay et al., 1991; Borrow et al., 1990; de Thé et al., 1990; Kakizuka et al., 1991; Rowley et al., 1977). Přibližně u 1–2 % pacientů s APL dochází ke vzniku variantních translokací, kdy je gen *RARα* fúzován s jiným partnerem než s *PML* (Liquori et al., 2020). Jedním z variantních fúzních genů je *NPM1::RARα* (Corey et al., 1994). Ačkoli některé studie vyhodnocují ATRA jako účinnou v terapii APL pacientů s přítomností NPM1::RARα, je její účinek u této formy onemocnění stále diskutován (Corey et al., 1994; Grimwade et al., 2000; Hummel et al., 1999; Kikuma et al., 2015; Nicci et al., 2005; Sanz et al., 2019; Xu et al., 2001). V této publikaci jsme charakterizovali vliv ATRA na expresi a lokalizaci NPM1 a na stabilitu jeho oligomerů jak u leukemických buněk pacientů s AML, tak u buněčných linií OCI-AML3 a OCI-AML2. Někteří autoři popisují degradaci mutNPM1 v důsledku působení ATRA a následné uvolnění wtNPM1 z heterooligomerů lokalizovaných v cytoplazmě a jeho přesun zpět do jádra (El Hajj et al., 2015; Martelli et al., 2015). Naše imunofluorescenční pozorování buněk ošetřených ATRA neprokázala výrazný úbytek mutNPM1, nicméně jsme zaznamenali jeho částečný přesun z cytoplazmy do jádra. Ve shodě s publikací *Martelli et al.* jsme též pozorovali změnu v počtu a tvaru jadérek buněk linie OCI-AML3 vyvolanou působením ATRA. Expresi přirozené i mutované formy NPM1 jsme dále stanovili pomocí WB, z nichž byla zjištěna mírně snížená hladina exprese obou variant NPM1 a zároveň vyšší frakce NPM1 v oligomerním uspořádání (zejména u buněk OCI-AML3).

Rozličné funkce a vnitrobuněčná lokalizace NPM1 jsou modulovány skrze jeho oligomerizační stav (Enomoto et al., 2006; Jian et al., 2009; Mitrea et al., 2014; Russo et al., 2021), přičemž zastoupení monomerů a oligomerů NPM1 je regulováno zejména jeho (de)fosforylací a interakcemi s jinými proteiny skrze jejich segmenty bohaté na přítomnost argininu (Mitrea et al., 2014). Monomery NPM1 se uplatňují zejména během poškození DNA a apoptózy (Russo et al., 2021), v průběhu S a M fáze buněčného cyklu (Chou and Yung, 1995). Předpokládáme, že vyšší podíl oligomerů v buňkách ošetřených ATRA souvisí s diferenciačním účinkem ATRA. Zvýšený počet buněk nacházejících se v G0/G1 fázi jsme potvrdili pomocí průtokové cytometrie. Vzhledem k pozorovanému efektu ATRA na lokalizaci mutNPM1 jsme toto léčivo využili také v experimentech s látkami navozujícími kolokalizaci proteinů NCL a mutNPM1 za účelem posouzení jejich případné interakce. Ani v tomto případě však nebyla mezi studovanými proteiny detekována žádná interakce.

Můj podíl na práci: (1) Transfekce exogenních proteinů (NCL a různých forem NPM1) do buněčných linií HEK-293T a HeLa; (2) provedení lokalizačních experimentů využívajících konfokální mikroskopii (live-cell imaging, imunofluorescence); (3) příprava buněčných lyzátů, provedení nat PAGE, semi-nat PAGE a SDS-PAGE, IP pomocí GFP/RFP-Trapu a vyhodnocení WB; (4) příprava obrázků a účast na psaní manuskriptu.

4.4 Publikace č. 4: NSC348884 cytotoxicity is not mediated by inhibition of nucleophosmin oligomerization

Shrnutí obsahu: Publikace diskutuje účinky léčiva NSC348884, které je používáno jako inhibitor oligomerizace NPM1. Ačkoli NSC348884 ovlivňuje také proliferaci, expresi p53 a apoptózu (Balusu et al., 2011; Y. Guo et al., 2014; Luo et al., 2017; Phi et al., 2019; Qi et al., 2008; Yu et al., 2021), řada studií jeho cytotoxický účinek připisuje právě narušené schopnosti NPM1 oligomerizovat (Hu et al., 2019; Phi et al., 2019; Reichert and Rotshenker, 2019). V dosavadních publikacích bylo působení NSC348884 na úrovni ovlivnění oligomerizace NPM1 analyzováno pomocí PAGE buněčných lyzátů získaných za nativních podmínek (Balusu et al., 2011; Qi et al., 2008; Yu et al., 2021). Naše výsledky z nat PAGE však nepotvrdily destabilizační efekt NSC348884 na komplexy oligomerů NPM1 u buněčných linií HL-60, MV4-11, KG-1, OCI-AML2, OCI-AML3, HEK-293T a HeLa bez ohledu na přítomnost mutNPM1. Rozhodli jsme se tedy účinek léčiva ověřit také *in vivo* metodami – konfokální mikroskopií a metodou FLIM-FRET – a rozšířit testování *in vitro* o IP využívající systém GFP/RFP-Trap. Funkčnost měřících postupů pro detekci oligomerů NPM1 byla ověřena jak pozitivními kontrolami (eGFP- a mRFP1-značenými formami wtNPM1 a mutNPM1), tak souborem negativních kontrol, tedy molekul NPM1 nesoucích mutace v oligomerizační doméně. Ty byly navrženy dle dat z publikací (Prinos et al., 2011), (Huang et al., 2013) a (Enomoto et al., 2006), v nichž jsou charakterizovány konkrétní oblasti a AA zbytky odpovědné za oligomerizaci NPM1. Jako první negativní kontroly byly připraveny konstrukty nesoucí NPM1 se záměnou p.Cys21Ala a p.Cys21Phe. *In vitro* byla metodou nat PAGE pozorována částečná destabilizace takovýchto oligomerních komplexů (Fig. 2), nicméně, jak bylo prokázáno v naší dřívější publikaci prostřednictvím metod fluorescenční mikroskopie, FLIM-FRET, PIE-ccN&B a IP, interakční schopnost těchto mutantů nebyla inhibována (Holoubek et al., 2018). Tvorbu heterooligomerů jsme potvrdili také v mikroskopických analýzách zaznamenávajících částečný přesun mRFP1-značených proteinů nesoucích mutaci v Cys21 do cytoplazmy v důsledku tvorby heterooligomerů s eGFP_mutNPM1 v buňkách HEK-293T (Fig. 1). Protože varianty p.Cys21Ala ani p.Cys21Phe proteinu NPM1 nebyly shledány jako vhodná negativní kontrola, byla připravena série konstruktů s rozsáhlejšími modifikacemi – NPM1 nesoucí delece prvních 25 (△25 NmutNPM1), 100 (△100 NmutNPM1) a 117 (△117 NmutNPM1) AA. Z publikace *Enomoto et al*. vyplývá, že NPM1 protein zkrácený na N-konci (NmutNPM1) není schopen oligomerizovat a je kompletně relokalizován do nukleoplazmy (vyjma varianty NPM1 o délce

25–294 AA, jehož oligomerizační vlastnosti i jadérková lokalizace mají zůstat částečně zachovány) (Enomoto et al., 2006). Pomocí konfokální mikroskopie jsme pozorovali přítomnost všech variant NmutNPM1 jak v jadérku, tak v nukleoplazmě, přičemž podíl nukleoplazmatické frakce se zvyšoval s rozsahem N-koncové delece. Výsledky získané metodou IP prokázaly, že ačkoli NmutNPM1 skutečně postrádají schopnost tvořit mezi sebou oligomerní komplexy, jejich interakce s přirozenou celodélkovou formou NPM1 zůstává do jisté míry zachována. Absence změny času dohasínání fluorescence donoru při použití *in vivo* metody FLIM-FRET též naznačuje neschopnost NmutNPM1 forem oligomerizovat. Mimoto jsme shledali, že tyto N-koncové delece usnadňují koprecipitaci jadérkových vazebných partnerů NPM1, NCL a FBL, což patrně souvisí s konformační změnou NmutNPM1 ovlivňující přístupnost vazebných míst pro dané interakční partnery. Potvrdili jsme rovněž výrazný úbytek proteinu p14Arf v precipitátech zkrácených forem, což odpovídá dřívějším pozorováním jiných autorů, že p14Arf interaguje s NPM1 skrze jeho oligomerizační doménu (Enomoto et al., 2006). Na základě naměřených dat byla jako adekvátní negativní kontrola pro další experimenty testující účinky léčiva NSC348884 vybrána dvojice △117 NmutNPM1 proteinů fúzovaných se dvěma různými fluorescenčními proteiny, eGFP a mRFP1.

Za účelem stanovení efektivní koncentrace NSC348884 byly na adherentních buněčných liniích HeLa, HEK-293T a několika liniích ustavených z buněk pacientů s AML provedeny testy viability a detekovány apoptotické markery (hladina p53 proteinu a aktivní forma kaspázy-3). V souladu s výsledky z předchozích publikací (Balusu et al., 2011; Qi et al., 2008) byla u některých buněčných linií ošetřených NSC348884 zaznamenána vyšší exprese p53 a v závislosti na dávce léčiva a době jeho působení také aktivace apopózy. V rozporu s daty naměřenými *Balusu et al.* naše experimenty na buněčných liniích OCI-AML2 a OCI-AML3, stejně tak jako na pacientských buňkách, neprokázaly, že by přítomnost mutace NPM1 byla spojena s vyšší citlivostí k léčivu NSC348884. Samotný účinek inhibitoru NSC348884 na oligomery NPM1 v buňkách HeLa a/nebo pouze HEK-293T byl sledován (1) konfokálním mikroskopem, (2) metodou FLIM-FRET, (3) IP a (4) technikou nat PAGE a semi-nat PAGE. Výsledky měření efektu NSC348884 pomocí jednotlivých metod jsou následující: (1) fluorescenční mikroskopií byla detekována nezměněná frakce cytoplazmaticky lokalizovaného mRFP1_wtNPM1 v důsledku zachovaných heterooligomerů mRFP1_wtNPM1-eGFP_mutNPM1 (podobné výsledky jsme získali i pro opačné značení, tedy kombinaci eGFP_wtNPM1 a mRFP1-značený mutNPM1 (mRFP1_mutNPM1)), (2) prodloužený čas

dohasínání fluorescence eGFP po vybělení akceptoru mRFP1 u párů eGFP_wtNPM1-mRFP1_wtNPM1 a eGFP_mutNPM1-mRFP1_mutNPM1 prokázal přetrvávající interakci mezi fluorescenčně značenými molekulami NPM1, (3) koprecipitace endogenní i exogenní formy NPM1 s příslušnou variantou NPM1 v buňkách kotransfekovaných eGFP_wtNPM1-mRFP1_wtNPM1 a eGFP_mutNPM1-mRFP1_mutNPM1 potvrdila přítomnost interakcí mezi jednotlivými variantami NPM1, (4) analýza lyzátů buněčné linie HEK-293T metodou nat PAGE, semi-nat PAGE a WB odhalila neporušené oligomerní komplexy eGFP_wtNPM1-mRFP1_wtNPM1, eGFP_mutNPM1-mRFP1_mutNPM1 a mRFP1_wtNPM1-eGFP_mutNPM1, což opět signalizuje žádný nebo zanedbatelný účinek léčiva NSC348884 na oligomerizaci NPM1. Lze tedy shrnout, že oligomerizace NPM1 byla studována několika vzájemně se doplňujícími přístupy, přičemž žádný z nich neprokázal inhibiční vliv léčiva NSC348884 na tvorbu oligomerů NPM1.

Zajímavým výsledkem této publikace je změna adhezivních vlastností buněk HeLa a HEK-293T po inkubaci s NSC348884. Snížená buněčná adheze byla zpočátku pozorována pomocí mikroskopie a poté charakterizována skrze měření elektrické impedance, které monituruje přilnavost adherentních buněk k povrchu elektrody. Jelikož změny impedance vyvolané léčivem NSC348884 měly podobný charakter jako změny dříve pozorované po přidání IPA-3, inhibitoru p21-aktivovaných kináz (Grebeňová et al., 2019), prostudovali jsme aktivitu a expresi vybraných regulačních proteinů aktinového cytoskeletu, p21-aktivované kinázy 1 (PAK1; p21-activated kinase 1) a kofilinu. Oba proteiny vykazovaly v důsledku působení NSC348884 změny v úrovni fosforylace (nižší úroveň fosforylace PAK1 na Ser144 a vyšší úroveň fosforylace kofilinu na Ser3), aniž by byla ovlivněna hladina jejich celkové exprese.

Můj podíl na práci: (1) Příprava vhodných variant NPM1 s mutací v NTD technikou molekulárního klonování a jejich transfekce do buněčných linií; (2) provedení lokalizačních experimentů využívajících konfokální mikroskopii; (3) příprava buněčných lyzátů, provedení nat PAGE, semi-nat PAGE a SDS-PAGE, IP pomocí GFP/RFP-Trapu a vyhodnocení WB; (4) příprava obrázků a účast na psaní a revizích manuskriptu.

5 DISKUZE

Ačkoli je AML s mutNPM1 klasifikována WHO jako samostatná kategorie (Arber et al., 2016), role NPM1 mutací v procesu leukemogeneze zůstává stále nejasná. K analýze mutNPM1 se využívá kombinace několika detekčních technik (Falini et al., 2010), přičemž ne všechny používané metodiky spolehlivě detekují přítomnost mutace (Venanzi et al., 2021). Cytoplazmatická lokalizace mutNPM1 je typickým nálezem u podtypu AML s mutNPM1 a má zřejmě zásadní vliv na vývoj a transformaci onemocnění (Bolli et al., 2007; Brunetti et al., 2017; Falini et al., 2009; Martelli et al., 2021; Pitiot et al., 2007; Venanzi et al., 2021). Tuto teorii podporuje skutečnost, že i v případě vzácných mutací NPM1 v jiném exonu než 12 a rozmanitých fúzí genu *NPM1* je výsledný produkt v různé míře zastoupen v cytoplazmě (Albiero et al., 2007; Bischof et al., 1997; Campregher et al., 2016; Chattopadhyay et al., 2014; Falini et al., 2021, 2006a; Mariano et al., 2006; Martelli et al., 2021, 2016; Martelli, 2018). Výjimku tvoří jeden popsaný případ, kdy NPM1 nesoucí mutaci v exonu 11 a zároveň 12 nebyl detekován v cytoplazmě, ale pouze v jádře, za což zřejmě zodpovídá chybějící NES běžně utvářený v CTD mutNPM1 (Pianta et al., 2009). Nukleoplazmatickou, nikoli cytoplazmatickou, lokalizaci lze předpokládat také u nově identifikovaného fúzního proteinu vytvořeného spojením *TRIP12* (z angl. *thyroid hormone receptor interactor 12*) a *NPM1* (Shiba et al., 2019). N-koncová vnitřně neuspořádaná oblast TRIP12, která zprostředkovává jadernou lokalizaci proteinu (Larrieu et al., 2020), je fúzována s CTD NPM1 vykazující vazebnou afinitu k NA a nesoucí NoLS. Koncentraci mutNPM1 v cytoplazmatické frakci stejně jako obsah wtNPM1 v jadérku ovlivňuje relativní zastoupení těchto variant v buňce, poněvadž v důsledku tvorby heterooligomerů dochází k přesunu části každého z obou partnerů do buněčných kompartmentů charakteristických pro lokalizaci druhé varianty (Bolli et al., 2009; Brodská et al., 2017, 2016a). *Bolli et al.* tuto situaci popisuje v expresním systému NIH-3T3, zatímco v pacientských blastech exprimujících mutNPM1 přesun mutované varianty NPM1 do jádra nezaznamenává. Dále tato práce ukazuje nejen cytoplazmatickou, ale také nukleoplazmatickou lokalizaci exogenního wtNPM1 v buňkách kotransfekovaných nadbytkem mutNPM1. V našich experimentech na buněčných liniích HEK-293T a HeLa transfekovaných plazmidy nesoucími mutNPM1 a wtNPM1 v poměru 8:1 však tato situace nenastala a wtNPM1 byl detekován v cytoplazmě a případně v jadérku (Kráčmarová, 2016). Vznik heterooligomerů mezi značenou mutovanou a značenou přirozenou formou NPM1 jsme pozorovali konfokálním mikroskopem u buněk NIH-3T3, HEK-293T a HeLa (viz publikace č. 1 a

(Kráčmarová, 2016)), přičemž poslední uvedená buněčná linie vykazovala velmi intenzivní fluorescenci mutNPM1 v jadérku (Kráčmarová, 2016). Vysoká jadérková exprese mutNPM1 byla patrná také v HeLa buňkách obsahujících pouze tento typ vektoru. Možné vysvětlení pro takto účinný přesun mutNPM1 do jadérka přinesly výsledky karyotypizace linie HeLa odhalující zmnožení oblasti 5q, ve které se nachází lokus pro *NPM1* (Macville et al., 1999). Interakci endogenního NPM1 s exogenní, přirozenou nebo mutovanou, formou NPM1 následně potvrdila imunoprecipitační analýza z buněčných lyzátů HEK-293T a HeLa (viz publikace č. 1). Exogenní wtNPM1 koprecipitoval endogenní NPM1 mnohem účinněji než exogenní mutované varianty NPM1. Jako možné vysvětlení tohoto pozorování jsme zpočátku uvažovali identickou lokalizaci, a tedy lepší přístupnost nemutovaných proteinů wtNPM1. Poněvadž jsme však u mutNPM1 typu A lokalizovaného převážně v cytoplazmě neshledali snížené množství koprecipitovaného endogenního NPM1 oproti mutNPM1 typu E, který se do značné míry nachází i v jadérku, tato hypotéza se nejevila jako pravděpodobná. Později jsme odhalili nižší stabilitu oligomerních komplexů tvořených molekulami mutNPM1 (viz publikace č. 3), což by mohlo být důvodem slabší koprecipitace endogenní formy NPM1. S nálezy konfokální mikroskopie korelovaly výsledky imunoblotu detekujícího rozdíly v poměru exprese endogenního a exogenního wtNPM1 v buněčných liniích HEK-293T a HeLa (viz publikace č. 1). Dále jsme popsali, že vnitrobuněčná distribuce proteinu NPM1 je ovlivněna nejen relativní koncentrací variant NPM1 přítomných v buňce, ale také konkrétním typem mutace NPM1 (viz publikace č. 1 a (Kráčmarová, 2016)). Publikace *Bolli et al.* upozorňuje na heterogenní lokalizaci wtNPM1 v blastech pacientů s mutNPM1 a jako možnou příčinu uvádí rozdíly v genové expresi mutované a zdravé alely *NPM1* (Bolli et al., 2009). Nedávná studie na souboru AML pacientů vskutku potvrzuje zvýšenou hladinu mRNA transkriptu z mutované alely *NPM1* oproti transkriptu z alely zdravé (Bailey et al., 2020). U vzorků vyšetřovaných v práci *Bolli et al.* však zřejmě nebyl stanoven konkrétní typ vyskytujících se mutací (Bolli et al., 2009), což může být další faktor sehrávající roli v rozmanité lokalizaci endogenního wtNPM1. Ve shodě s jinou studií téhož autora (Bolli et al., 2007) jsou naše data naměřená ve vzorcích leukemických buněk od pacientů s AML i v buněčné linii HEK-293T, ze kterých vyplývá, že mutace NPM1 vedoucí k deleci celého NoLS způsobují jeho účinnější translokaci do cytoplazmy oproti mutacím NPM1, které NoLS poškozují pouze částečně. Vzhledem k tvorbě heterooligomerů obsahujících wt a mutovanou formu NPM1 se tedy pravděpodobně zastoupení NPM1 v jadérku odvíjí také od typu přítomné mutace. Dalo by se proto očekávat, že rozdílné množství

wtNPM1 soustředěného v jadérku leukemických buněk bude mít vliv na prognózu nemocných. V odborných kruzích však nepanuje jednotný názor na význam konkrétních typů mutací NPM1 v prognóze onemocnění (Alpermann et al., 2016; Heiblig et al., 2019; Koh et al., 2009; Park et al., 2012; Pastore et al., 2014; Sciumè et al., 2019; Selim et al., 2016). Zajímavý poznatek týkající se této kontroverze přináší nedávný výzkum *Tregnago et al.* porovnávající soubor mutovaných variant NPM1 s kompletně a s částečně deletovaným NoLS. U buněk z první uvedené skupiny byla ve srovnání s druhou skupinou detekována vyšší míra exprese *HOXA* genů a nižší hladina p53 promítající se do snížené citlivosti nádorových buněk k chemoterapii nebo k inhibitoru antiapoptotického proteinu BCL-2, Venetoclaxu (Tregnago et al., 2021). Poněvadž nadměrná exprese *HOX* byla identifikována jako ukazatel nepříznivé prognózy AML s mutNPM1 (Nagy et al., 2019), autoři publikace *Tregnago et al.* spekulují, že typ mutace NPM1 může poskytnout informaci o očekávaném průběhu onemocnění (Tregnago et al., 2021).

Publikace č. 2 přináší nové metodické přístupy studia oligomerizace NPM1 umožňující potvrdit nebo vyvrátit přítomnost interakce mezi dvěma kolokalizujícími proteiny v živých buňkách. Za účelem studia vlivu inhibitorů oligomerizace NPM1 jsme zavedli nové metody PIE-ccN&B a FLIM-FRET umožňující získat více informací o oligomerizaci NPM1 *in vivo*, a následně jsme hledali vhodnou negativní kontrolu s AA sekvencí co nejpodobnější wtNPM1, ale s poškozenou schopností oligomerizace (viz publikace č. 2). Záměna Cys21 v proteinu NPM1 za Phe či Trp poškozuje dle autorů *Prinos et al.* tvorbu oligomerů NPM1, přičemž ke stanovení oligomerizačního stavu NPM1 využili metod 10% SDS-PAGE (s buněčnými lyzáty či purifikovaným rekombinantním proteinem NPM1 sklizenými do Laemmliho pufru bez povaření) a WB (Prinos et al., 2011). Podobně bylo popsáno narušení tvorby oligomerů v případě záměny Cys21 za Ala (Huang 2013). Efekt těchto substitucí jsme ověřovali u NPM1 mutantů p.Cys21Ala a p.Cys21Phe pomocí technik PIE-ccN&B, FLIM-FRET a IP (viz publikace č. 2) a později také prostřednictvím konfokální mikroskopie, nat PAGE a semi-nat PAGE (viz publikace č. 4). Žádná z námi provedených metod nepotvrdila úplnou ztrátu schopnosti oligomerizace NPM1 v důsledku mutace v Cys21, nicméně experimenty *in vitro* zaznamenaly nižší stabilitu oligomerních komplexů tvořených těmito mutovanými variantami (viz publikace č. 4). Nesoulad mezi výsledky elektroforéz prováděných námi a *Prinos et al.* může být zapříčiněn odlišnými podmínkami PAGE – ačkoli v publikaci *Prinos et al.* lýze buněk probíhala bez využití vysokých teplot, lyzáty byly naředěny v Laemmliho pufru (s obsahem SDS a

β-merkaptoetanolu) a proteiny byly následně separovány v gelu obsahujícím SDS. Tyto podmínky jsou blízké těm, které v našich experimentech označujeme jako seminativní. Dle našich výsledků seminativní podmínky indukují rozpad NPM1 oligomerních komplexů obsahujících p.Cys21Ala nebo p.Cys21Phe, nicméně za nativních podmínek, tedy zcela bez použití denaturujícího SDS a jen s nízkou koncentrací redukujícího dithiotreitolu, zůstávají tyto oligomery alespoň zčásti zachovány (viz publikace č. 4). Uvedené rozdíly v podmínkách přípravy vzorků vysvětlují i skutečnost, že jsme v rozporu s *Prinos et al.* detekovali prostřednictvím IP schopnost NPM1 p.Cys21Phe interagovat s endogenním NPM1 (publikace č. 2). Rozhodli jsme se tedy připravit varianty NPM1 s kratšími nebo rozsáhlejšími delecemi v NTD, až po deleci celé této domény. Tyto delece dle literatury vedou ke ztrátě schopnosti proteinu oligomerizovat a interagovat s endogenní formou NPM1 a k jeho relokalizaci do nukleoplazmy (Enomoto et al., 2006). Ačkoli jsme též zaznamenali uvolnění těchto deletovaných variant do nukleoplazmy, stále jsme, na rozdíl od *Enomoto et al.*, pozorovali jejich zřetelnou akumulaci v jadérku (viz publikace č. 4). Roli zde pravděpodobně sehrává přítomnost NoLS a domény zodpovědné za vazbu k NA. Dle našich výsledků NmutNPM1 sice netvoří homooligomerní komplexy, jsou ale schopny interagovat s endogenním i exogenním wtNPM1 (viz publikace č. 4). S rozsáhlejší delecí NTD NPM1 množství koprecipitovaného endogenního NPM1 klesá. Také tato interakce zřejmě přispívá k udržení jadérkové lokalizace NmutNPM1, přičemž její síla odpovídá poměrnému zastoupení jednotlivých variant NmutNPM1 v jadérku. Možné vysvětlení interakce NmutNPM1 s wtNPM1 navzdory částečné či úplné absenci oligomerizační domény spočívá v charakteru interakce NmutNPM1 a dalších jadérkových proteinů NCL a FBL (viz publikace č. 4). Na základě odlišné struktury NmutNPM1, a tedy pravděpodobně odlišné přístupnosti interakční oblasti pro tyto dva jadérkové proteiny, dochází v buněčných lyzátech k výrazně vyšší koprecipitaci NCL a FBL s NmutNPM1 než s wtNPM1. Poněvadž wtNPM1 patří mezi interakční partnery NCL (Li et al., 1996) a FBL (Amin et al., 2008; Holoubek et al., 2021), spekulujeme, že se jeho asociace s NmutNPM1 uskutečňuje právě prostřednictvím komplexů s proteiny NCL a FBL. Tyto proteinové komplexy jsou pak zřejmě příliš velké na to, aby pronikaly do pórů polyakrylamidového gelu, takže analytické techniky nat PAGE a následný WB je nezachytí (viz publikace č. 4). Zajímavý poznatek přineslo studium vazby mezi NmutNPM1 a wtNPM1 pomocí metod FLIM-FRET a IP ukazující, že tuto interakci lze prokázat pouze v jednom uspořádání (viz publikace č. 4). Pokud je wtNPM1 použit jako akceptor při procesu FRET nebo je koprecipitován společně

s NmutNPM1, potvrzuje se existence interakce. V opačném případě, tedy je-li wtNPM1 donorem pro přenos fluorescenční energie nebo pokud je přímo precipitován z buněčného lyzátu, jeho interakci s NmutNPM1 nedetekujeme. Domníváme se, že heterooligomery mezi těmito dvěma partnery jsou složeny zejména z podjednotek wtNPM1, které zajišťují dostatečnou stabilitu komplexu. Na jednu molekulu NmutNPM1 tak teoreticky připadá několik molekul wtNPM1, jejichž přítomnost je detekovatelná IP a WB a jejichž fotovybělení jakožto akceptoru ve FRET experimentech způsobuje prodloužení doby dosvitu fluorescence donoru čili NmutNPM1.

NPM1 a NCL patří mezi proteiny se zásadní strukturální rolí v architektuře jadérka a zároveň zastávají komplexní funkce jako je regulace buněčného dělení či apoptózy a reakce na stresové faktory (Brodská et al., 2019; Tajrishi et al., 2011). Ačkoli se mutace NPM1 téměř výhradně vyskytují v jeho C-koncové oblasti (Borrow et al., 2019; Falini et al., 2005), která není vazebným místem pro NCL (Li et al., 1996), jejich důsledkem je narušení schopnosti takto mutovaného proteinu interagovat s NCL (viz publikace č. 3). V sérii experimentů využívajících cytotoxických léčiv pro přesun mutNPM1 a NCL do nukleoplazmy jsme testovali předpoklad, že tvorba komplexu mutNPM1-NCL by mohla být obnovena jejich relokalizací do společného buněčného kompartmentu. Vazba mezi těmito proteiny však ani při takovýchto podmínkách detekována nebyla, což naznačuje, že absence interakce mutNPM1-NCL není způsobena odlišnou vnitrobuněčnou lokalizací obou partnerů. Změna vazebných schopností mutNPM1 je tedy mnohem pravděpodobněji důsledkem odlišné konformace jeho CTD (Di Natale et al., 2019; Grummitt et al., 2008). S ohledem na podobnou lokalizaci vazebných míst proteinu NPM1 pro p53 a pro NCL (186–259 AA (Colombo et al., 2002), respektive 242–269 AA (Lambert and Buckle, 2006) versus 187–245 AA (Li et al., 1996)) přináší zajímavé výsledky studie *Holoubek et al.*, ve které je prokázáno, že mutace NPM1 neznemožňuje jeho interakci s p53 (Holoubek et al., 2021). Interakce p53 s variantami NPM1 s deletovanou NTD navíc poukazuje na to, že tvorba komplexu mezi těmito dvěma proteiny je zároveň nezávislá na oligomerizačních vlastnostech NPM1. Dynamický charakter interakce mezi p53 a mutNPM1 s deletovanou NTD ale napovídá, že v tomto případě je NTD důležitá pro stabilitu utvořeného komplexu (Holoubek et al., 2021). Podle studie *Tregnago et al.* (Tregnago et al., 2021) se množství cytoplazmaticky lokalizovaného p53 v důsledku mutace NPM1 odvíjí od jejího typu. K výsledkům této práce je ale potřeba přistupovat s vědomím, že v experimentech byly použity buněčné linie s nestandardní expresí p53 a výsledky na buňkách s wtp53 se mohou lišit. Zcela

jednoznačně jsme však prokázali vliv typu NPM1 mutace na množství wtNPM1, který je díky účasti v heterooligomerech s mutNPM1 přemístěn z jadérka do cytoplazmy (viz publikace č. 1). Mutace NPM1, které neruší jeho NoLS kompletně, ponechávají mutovaný protein částečně v jadérku a v komplexech s ním i jeho interakční partnery. Lze tedy očekávat, že v těchto buňkách bude biologická aktivita NPM1 méně ovlivněna a že i funkce p53 bude v důsledku vyšší jadérkové frakce proteinu NPM1 méně zasažena (Tregnago et al., 2021). K rozdílné citlivosti AML buněk s mutNPM1 k použité léčbě může tak přispívat účinnější apoptóza nádorových buněk nesoucích mutace směřující NPM1 do cytoplazmy pouze částečně (Tregnago et al., 2021). Zvýšená rezistence buněk linie OCI-AML3 k léčivům indukujícím p53-dependentní apoptózu (např. Aktinomycin D (Brodska et al 2016), NSC348884 (publikace č.4), nebo selinexor (Holoubek et al 2021)) tedy může souviset s narušením dráhy p53, vyvolaným aberantní delokalizací p53 do cytoplazmy. Potenciální faktor ovlivňující pozdější nástup apoptózy vyvolané léčivy u buněčné linie OCI-AML3 však může představovat také relativně nižší hladina p53 v těchto buňkách oproti OCI-AML2 (viz publikace č. 4 a (Holoubek et al., 2021). Na rozdíl od exprese p53 je relativní hladina NCL v buňkách pacientů s AML a nádorových linií OCI-AML2 a OCI-AML3 stabilní, bez ohledu na přítomnost mutace NPM1 (viz publikace č. 3). Tím v buňkách s mutNPM1 dochází k vychýlení poměru relativních koncentrací NCL a NPM1 schopného interagovat s NCL. Zvýšená exprese NCL bývá detekována v různých typech nádorových tkání (Balça-Silva et al., 2018; X. Guo et al., 2014; Qiu et al., 2013; Wolfson et al., 2016; Xu et al., 2016; Z. Xu et al., 2012; Yan et al., 2016) včetně leukemických buněk (Marcel et al., 2017; Otake et al., 2007; Shen et al., 2014), přičemž nadměrná hladina jeho transkriptu je u AML pacientů starších 60 let ukazatelem horší prognózy (Marcel et al., 2017). Jedním ze základních rysů nádorové buňky je zvětšený nárok na tvorbu proteinů, což vyžaduje také zvýšenou transkripci rDNA (Hein et al., 2013). Abnormální množství NCL, známého regulátoru syntézy rRNA (Bouche et al., 1984; Egyhazi et al., 1988; Ginisty et al., 1998; Roger et al., 2003; Sipos and Olson, 1991), tedy může podpořit nádorovou transformaci buněk hyperaktivací biogeneze ribozomů a dále např. stimulací exprese protiapoptotických proteinů BCL-2 a AKT serin/threonin kinázy 1 či potlačením translace tumor supresoru p53 (Abdelmohsen and Gorospe, 2012). Lze proto očekávat, že změna poměru mezi hladinou wtNPM1 a hladinou NCL v buňkách s mutNPM1 bude mít dopad na kooperaci obou proteinů a může tak být jedním z faktorů přispívajících k rozvoji leukémie.

Poněvadž oligomery sestavené z molekul mutNPM1 jsou méně stabilní než oligomery wtNPM1 (viz publikace č. 3), vhodně zvolená koncentrace inhibitoru oligomerizace NPM1 může umožnit rozrušení oligomerních struktur mutNPM1 za současného zachování komplexů nesoucích wtNPM1. NSC348884, jehož efekt jsme podrobně analyzovali v publikaci č. 4, je v řadě studií používáno pro destabilizaci oligomerů NPM1. Zaznamenané změny buněčných dějů a vlastností jsou pak dávány do souvislosti s inhibicí oligomerizace NPM1. Vedle rozpadu NPM1 oligomerních komplexů (Balusu et al., 2011; Qi et al., 2008; Yu et al., 2021) patří k nejčastěji pozorovaným efektům NSC348884 snížená exprese NPM1 (Luo et al., 2017; Phi et al., 2019), inhibice proliferace nádorových buněk (Phi et al., 2019; Qi et al., 2008) a jejich apoptóza (Balusu et al., 2011; Y. Guo et al., 2014; Luo et al., 2017; Phi et al., 2019; Qi et al., 2008; Yu et al., 2021). Kromě toho byl v buňkách v odezvě na NSC348884 zaznamenán přesun NPM1 do nukleoplazmy a částečně i do cytoplazmy a zároveň přechodné zvýšení hladiny reaktivních kyslíkových radikálů (ROS; reactive oxygen species) a pokles exprese antioxidačního enzymu PRDX6 (z angl. *peroxiredoxin-6*) vysoce exprimovaného u různých typů nádorů (Liu et al., 2017). V souvislosti se známou podpůrnou funkcí NPM1 v procesu replikace virové NA (Duan et al., 2014; Huang et al., 2001; Mai et al., 2017; Okuwaki et al., 2001a; Shi et al., 2017; Song et al., 2021; Tsuda et al., 2006; Zhou et al., 2020) přináší zajímavé zjištění studie *Abraham et al.*, dle které NPM1 agreguje v cytoplazmě buněk napadených virem Chikungunya (CHIKV; Chikungunya virus) a brzdí jeho replikaci (Abraham et al., 2017). Popisovaná agregace NPM1 je v buňkách infikovaných CHIKV údajně inhibována NSC348884, následkem čehož dochází k účinnější replikaci viru a zvýšení virové zátěže. Nicméně na snímcích z konfokálního mikroskopu, které mají tuto skutečnost ilustrovat, je detekován wtNPM1 v cytoplazmě i v intaktních buňkách, v nichž by měl být lokalizován v jadérkách (Abraham et al., 2017). Z našich interních srovnávacích analýz souboru několika protilátek proti NPM1 od Santa Cruz Biotechnology vyplývá, že protilátka uvedená v této publikaci pro detekci NPM1 v sekci Materiál a Metody není pro imunofluorescenční zobrazování vhodná (Barbora Brodská, ústní sdělení). Popisované účinky NSC348884 na podporu replikace virové DNA nelze tedy jednoznačně připsat právě změnám v agregaci NPM1. Vedle synergického působení NSC348884 a běžně používaného chemoterapeutika DOX na viabilitu nádorových buněk (Qi et al., 2008) bylo pozorováno, že NSC348884 může potlačit mechanismus rezistence nádorových buněk k protinádorové léčbě prostřednictvím snížené hladiny glykoproteinu-P, jehož expresi NPM1 ovlivňuje (Luo et al., 2017). V posledních letech se stále rozšiřuje spektrum popsaných

účinků NSC348884 připisovaných jeho údajné schopnosti indukovat rozpad oligomerů NPM1 (Hu et al., 2019; Reichert and Rotshenker, 2019). V dosavadních studiích testujících destabilizační efekt NSC348884 je však pro detekci oligomerů NPM1 využita pouze jedna metoda, a to polyakrylamidová gelová elektroforéza buněčných lyzátů získaných za nativních (nedenaturačních) podmínek, tj. v nepřítomnosti SDS a bez ošetření vysokými teplotami (Balusu et al., 2011; Qi et al., 2008; Yu et al., 2021). Lyzáty jsou následně nanášeny do gelu s různým obsahem SDS (0–12 %) a po přeblotování na membránu jsou oligomerní i monomerní formy NPM1 detekovány specifickými protilátkami proti NPM1. Uváděné výsledky se liší jak polohou, ve které jsou detekovány oligomery (v rozsahu 120–200 kDa), tak způsobem detekce monomerů (koncentrace SDS v gelu, současně s oligomery nebo odděleně na jiném gelu apod.). Z popisu metody detekce oligomerů NPM1 ve studii *Yu et al.* vyplývá, že PAGE probíhala v gelu obsahujícím SDS, čili za seminativních podmínek, kdy podle našich výsledků dochází k částečnému rozpadu oligomerů na monomery (viz publikace č. 3 a č. 4), jejichž hladina však v této studii není analyzována (Yu et al., 2021). Imunobloty z nat PAGE v publikaci *Qi et al.* opět zachycují snižující se množství oligomerů NPM1 u buněk ošetřených NSC348884, nicméně i zde chybí zaznamenaný nárůst monomerů NPM1 (monomery NPM1 byly analyzovány jiným imunoblotem po separaci na semi-nat PAGE elektroforéze a i jejich exprese je po ošetření NSC348884 nižší než v kontrole) (Qi et al., 2008). Zaznamenaný pokles hladiny NPM1 v důsledku působení NSC348884 tedy může odrážet sníženou celkovou expresi NPM1, kterou kromě těchto dvou studií popisují i další publikace (Luo et al., 2017; Phi et al., 2019). Při pohledu na marker molekulových hmotností (MW; molecular weight) v polyakrylamidovém gelu bez SDS v publikaci *Qi et al.* a *Balusu et al.* vyvstává otázka správného určení pozic jednotlivých bandů, poněvadž oligomerní komplexy NPM1 jsou v poloze odpovídající struktuře trimerů (zhruba 120 kDa) (Balusu et al., 2011; Qi et al., 2008). Oproti tomu *Yu et al.* detekuje oligomery NPM1 při očekáváné velikosti okolo 200 kDa, což odpovídá jeho pentamerní formě (Yu et al., 2021). Námi stanovená velikost komplexů NPM1 okolo 360 kDa (viz publikace č. 2 a č. 4) koresponduje s dříve publikovanou hodnotou 358 kDa (Okuwaki et al., 2012), což by mohlo souhlasit se dvěma asociovanými pentamerickými cykly NPM1 (Lee et al., 2007). Samostatný námět pro další studii přináší vyšší MW oligomerních komplexů ve většině buněčných linií ošetřených NSC348884 oproti MW oligomerů v inhibitorem neošetřených kontrolních buňkách (viz publikace č. 4). Jako možné vysvětlení nabízíme úvahu, že NPM1 podléhá vlivem NSC348884 posttranslačním modifikacím, které

mohou měnit MW daného proteinu (Mocanu et al., 2009). Pro analýzu inhibičního účinku NSC348884 na oligomerizaci NPM1 jsme vedle nat PAGE a semi-nat PAGE využili fluorescenční mikroskopii, FLIM-FRET a IP, přičemž žádná z uvedených metod nepotvrdila schopnost tohoto léčiva destabilizovat oligomery NPM1 (viz publikace č. 4). Nepozorovali jsme ani popisovanou změnu vnitrobuněčné distribuce NPM1 navozenou v důsledku působení tohoto léčiva (Liu et al., 2017). Neměnná lokalizace NPM1 v našich experimentech s NSC348884 může souviset s dobou působení tohoto inhibitoru – zatímco *Liu et al.* sledoval přesun NPM1 z jadérka do cytoplazmy po 24 hodinách od přidání léčiva, působení NSC348884 v našich pokusech vedlo po 2 hodinách k uvolnění ošetřených buněk z podkladu (viz dále v textu). Ve shodě s jinými publikacemi (Balusu et al., 2011; Y. Guo et al., 2014; Luo et al., 2017; Phi et al., 2019; Qi et al., 2008; Wang et al., 2020; Yu et al., 2021) jsme ale zaznamenali apoptózu buněk ošetřených NSC348884, u buněk s funkčním p53 doprovázenou podstatným zvýšením jeho proteinové exprese. Na základě výsledků řady studií se tedy NSC348884 jeví jako perspektivní induktor apoptózy, k níž pravděpodobně přispívá vyvolání oxidativního stresu. Otázkou však zůstává jeho selektivní funkce v indukci apoptózy. Zatímco *Balusu et al.* pozoroval, že NSC348884 preferenčně spouští apoptózu v buňkách nesoucích mutNPM1 (linie OCI-AML3), v našem souboru byla tato buněčná linie jednou z nejméně citlivých k ošetření NSC348884 (viz publikace č. 4). Monitorování živých buněk konfokálním mikroskopem a měření elektrické impedance v průběhu působení NSC348884 odkrylo novou biologicky významnou vlastnost tohoto léčiva, kterou je schopnost narušovat přirozenou adhezi buněk (viz publikace č. 4). Jedním z nalezených cílů působení NSC348884 jsou p21-aktivované kinázy, jejichž aktivita je důležitá pro veškeré děje vyžadující práci cytoskeletu a jeví se také jako klíčová pro přežití leukemických buněk (Kuželová et al., 2021b). Dle našich analýz NSC348884, podobně jako inhibitor IPA-3 (Grebeňová et al., 2019), působí velmi rychlé uvolnění adherentních buněk z podkladu a snižuje kinázovou aktivitu PAK1. Aktivovaná PAK1 prostřednictvím LIM kinázy například inhibuje činnost kofilinu, proteinu depolymerizujícího aktin, což vede ke stabilizaci aktinových vláken (Condeelis et al., 2005). Dynamika aktinového cytoskeletu je nezbytným předpokladem nejen pro úspěšný průběh mitózy, ale také pro buněčnou motilitu a invazivitu či angiogenezi (Li and Wang, 2020). Zaznamenané změny buněčné adheze vyvolané působením NSC348884 korelují s údaji nedávné publikace *Hu et al.*, která prokazuje asociaci jadérkového stresu s implantací embrya do endometria (Hu et al., 2019). Jelikož je NPM1 připisována úloha v regulaci buněčné odpovědi na stres (Kurki et al., 2004; Yang et al., 2016;

Yao et al., 2010), zaměřila se tato práce na jeho roli v uhnízdění embrya do děložní sliznice. V rámci sledování buněčné lokalizace a exprese NPM1 autoři testovali také inhibitor NSC348884 a zjistili, že jeho působení snižuje počet míst k implantaci. Jedním z regulačních mechanismů pohybových procesů spjatých s embryogenezí, zánětlivou reakcí, hojením ran a tvorby metastáz je signální dráha kinázy regulované extracelulárním signálem (ERK; extracellular signal-regulated kinase) (Tanimura and Takeda, 2017). Úloha aktivace ERK byla popsána také v buněčné adhezi (Tanimura and Takeda, 2017). Zajímavým výsledkem vědecké skupiny *Liu et al.* je pak zjištění, že NSC348884 snižuje expresi fosforylované ERK (Liu et al., 2017). Tato zjištění poskytují nový pohled na působení NSC348884 a nasvědčují jeho schopnosti narušení buněčné adheze. Závěrem lze shrnout, že naše analýza vlivu inhibitoru NSC348884 na oligomerizaci NPM1 nepotvrdila výsledky doposud publikovaných studií a podtrhla význam kombinování odlišných výzkumných přístupů. Soubor metod, které prezentujeme v publikaci č. 4, představuje účinný způsob identifikace inhibiční aktivity kandidátních látek cílených na oligomerizaci NPM1. Kromě vlastního testování účinnosti NSC348884 na oligomery NPM1 bylo cílem optimalizovat koncentraci léčiva tak, aby nedocházelo k bezprostřední apoptóze a bylo tak dosaženo spolehlivých výsledků. Řada studií interpretující pozorované efekty NSC348884 jako následek inhibice oligomerizace NPM1 neprokazuje žádnou metodou, že k této inhibici vskutku dochází (Abraham et al., 2017; Y. Guo et al., 2014; Hu et al., 2019; Liu et al., 2017; Luo et al., 2017; Reichert and Rotshenker, 2019). Výsledky jejich experimentů tak pravděpodobně odrážejí alternativní regulaci buněčných pochodů, například tvorbu ROS nebo deregulaci adhezních mechanismů.

6 SHRNUTÍ

Tato dizertační práce analyzuje důsledky dvou typů mutací NPM1 na jeho oligomerizaci, lokalizaci a některé interakce. Jedním typem jsou specifické C-koncové mutace vyskytující se jako nejčastější geneticky podmíněná aberace u AML pacientů s NK, druhým typem jsou bodové mutace nebo delece v N-terminální doméně NPM1 simulující efekt případných inhibitorů NPM1 oligomerizace, které by bylo možno využít k léčbě pacientů s mutNPM1. Práce dále zkoumá účinky léčiv NSC348884 a ATRA na tvorbu oligomerů NPM1. Naše poznatky jsou prezentované ve čtyřech publikacích uvedených v kapitole 4 Výsledky.

Vytyčené cíle dizertační práce byly splněny a získané výsledky jsou následující:

1) Potvrdili jsme asociaci mezi typem C-koncové mutace NPM1 a lokalizací mutovaného proteinu a zaměřili se na stabilitu a tvorbu oligomerů přirozené i mutované formy NPM1.
   - potvrdili jsme interakci mezi exogenní (přirozenou i mutovanou) a endogenní formou NPM1
   - detekovali jsme nižší stabilitu oligomerních komplexů tvořených mutovanou variantou NPM1 ve srovnání s oligomery wtNPM1
2) Zavedli a optimalizovali jsme pro naše podmínky řadu metod pro detekci oligomerů NPM1: nativní a seminativní elektroforézu, imunoprecipitaci, konfokální mikroskopii a metody časově rozlišené fluorescence.
3) Zjistili a prokázali jsme, že v důsledku C-koncové mutace NPM1 je inhibována interakce NPM1 a NCL, a to i přesto, že sekvence NPM1 účastnící se vazby s NCL není C-terminální mutací zasažena. Exprese NCL v buňkách exprimujících mutNPM1 zůstává nezměněna, čímž dochází ke změně relativních koncentrací obou proteinů, a to může mít vliv na buněčné procesy, ve kterých tyto proteiny spolupracují.
4) Působení léčiv ActD, LmB či ATRA směřujících alespoň částečně oba interakční partnery, mutNPM1 a NCL, do stejného buněčného kompartmentu neobnovilo tvorbu komplexu mutNPM1-NCL, což naznačuje, že důvodem absence interakce mezi mutNPM1 a NCL zřejmě není fyzická separace těchto proteinů. Dále jsme pozorovali stabilizační efekt ATRA na oligomery NPM1 a také přesun mutNPM1 do jádra a mírně sníženou celkovou expresi NPM1 v důsledku jejího působení. Změny lokalizace

mutNPM1 po působení ActD a LmB odpovídaly dřívějším pozorováním a vliv těchto dvou léčiv na oligomerizaci NPM1 jsme nezaznamenali.

5) Charakterizovali jsme oligomerizaci, stabilitu a lokalizaci NPM1 s různě rozsáhlými delecemi v N-terminální doméně (△25 NmutNPM1, △100 NmutNPM1 a △117 NmutNPM1) a NPM1 nesoucího bodovou mutaci v Cys21 (p.Cys21Ala a p.Cys21Phe).
   - substituce Cys21 za Phe či Ala neinhibuje oligomerizaci NPM1, ale ovlivňuje stabilitu oligomerů (zejména p.Cys21Phe)
   - NPM1 p.Cys21Ala a p.Cys21Phe vykazují jadérkovou lokalizaci stejně jako wtNPM1
   - delece oligomerizační domény u NPM1 (△117 NmutNPM1) znemožní tvorbu homooligomerů, ale přítomnost nízké koncentrace △117 NmutNPM1 neinhibuje tvorbu komplexu s wtNPM1
   - všechny deletované varianty NmutNPM1 jsou přítomny jak v jadérku, tak v nukleoplazmě, v níž poměrné zastoupení dané varianty roste s rozsahem delece NTD
   - interakce NPM1 s dalšími jadérkovými proteiny, NCL a FBL, je delecí NTD posílena
6) Popsali jsme účinky léčiva NSC348884, jehož velmi důležitou uváděnou vlastností je inhibice oligomerizace NPM1.
   - potvrdili jsme proapoptotický účinek NSC348884
   - s využitím zavedených metodických přístupů rozporujeme schopnost NSC348884 inhibovat oligomerizaci NPM1
   - detekovali jsme novou vlastnost NSC348884, kterou je schopnost narušení adhezivity buněk

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8 PŘÍLOHY

8.1 Příloha č. 1: Localization of AML-related nucleophosmin mutant depends on its subtype and is highly affected by its interaction with wild-type NPM1

PLOS ONE

RESEARCH ARTICLE

Localization of AML-related nucleophosmin mutant depends on its subtype and is highly affected by its interaction with wild-type NPM

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Abstract

Mutations of the gene for nucleophosmin (NPM1) are the most frequent genetic aberration in patients with acute myeloid leukemia (AML). The mechanism of leukemic transformation in this leukemia subtype is not fully understood, but aberrant cytoplasmic localization of mutated NPM (NPMmut) is widely considered as an important factor for leukemia manifestation. We analyzed the subcellular localization of three types of NPM with a C-terminal mutation (A, B and E). Genes for the individual NPM forms were fused with a gene for one of fluorescent protein variants in plasmids, which were transfected into three cell lines with different endogenous NPM expression. Subcellular localization of the fluorescent protein-labeled NPM was further correlated with the relative expression of all NPM forms. We confirmed a high cytoplasmic expression of NPMmutA and NPMmutB whereas a substantial fraction of NPMmutE was found to be localized in nucleoli. Moreover, we revealed that the localization of fluorescently labeled NPM is affected by the interaction between various forms of the protein.

Introduction

The phosphoprotein nucleophosmin (NPM) is an abundant protein located mainly in the nucleolus, although it shuttles between the nucleolus, the nucleus and the cytoplasm. It regulates many cellular processes, mainly the ribogenesis [1], centrosome duplication control [2] and apoptosis [3,4]. Mutation of the *NPM1* gene is the most frequent genetic aberration in AML and generally causes NPM relocation from the nucleolus into the cytoplasm [5]. The mistargeting is caused by mutations in exon 12 of the *NPM1* gene leading to the loss of tryptophan W288 and/or W290 at the C-terminus of the resulting protein [6]. The mutations highly compromises the nucleolar localization signal (NoLS) and, moreover, the protein acquires an extra nuclear export signal (NES) in addition to two NESes already present in its N-terminal domain [7]. Specific NPM mutations are characteristic for about 60% of adult AML with the normal karyotype [8] and are associated with good response to induction therapy [9]. The most frequent AML-related NPM mutation type (type A) occurs in 75±80% of adult AML patients with NPM mutation [5,9±11]. The resulting mutated protein (NPMmutA) lacks both

tryptophans W288 and W290 and it has the most frequent NES motif L-xxx-V-xx-V-x-L [12]. Bolli et al [13] identified six different NES motifs with various exporting efficiency associated with C-terminal mutations. The strongest NES motifs were associated with NPM mutants retaining W288 that drives the NPM into the nucleolus. The authors concluded that a strong NES motif balancing the force of W288 allows for the export of NPMmut into the cytoplasm, and that NPM translocation might be critical for leukemogenesis. All AML-related NPM mutations reported to date were heterozygous, i.e. the patients were heterozygous for the mutation and retained a wild-type allele [5,9,10]. Homozygous Npm1 mutant knock-in mice were reported to show embryonic lethality [14].

The impact of the mutation type on survival characteristics was widely examined and the results of individual studies varied: while no difference in the overall survival (OS) and in the disease-free survival (DFS) was observed by Pastore et al. [15], other researchers reported either better or worse outcome for patients with NPMmutA vs. patients with mutations of other type [16,17]. The role of different types of *NPM1* mutation, either individually or in the presence of other common gene mutations was suggested to be essential also for childhood AML prognosis [18]. However, these studies generally compared the group of patients with the most frequent mutational type A versus a merged group of patients with other types of mutation. We believe that if the cytoplasmic localization of NPM is critical for leukemogenesis, the difference should be searched between types causing different subcellular localization. We thus compared the subcellular distribution of NPMmutA with that of NPMmut type B, which differs from the type A only in one aminoacid (L289M) and with the type E, which retains W288 and has the strongest NES motif, L-xxx-L-xx-V-x-L [19].

NPM conformation exhibits monomer±pentamer equilibrium, which is modulated by post-translational modifications, in particular by phosphorylation, and by protein binding [20], the pentamers being formed through the domain located at the N-terminus of the protein [21]. This domain is also responsible for the majority of interactions of NPM with various proteins [1,22]. It was reported, that the ability to oligomerize is, at least in part, maintained in C-terminal mutants [23]. Falini et al [24] suggested that the increased nucleophosmin export into the cytoplasm probably perturbs multiple cellular pathways by loss-of-function (delocalization of NPM nucleolar interactors into the cytoplasm) and/or gain-of-function mechanisms. Balusu et al [25] demonstrated that AML cells expressing mutated NPM are more sensitive to disruptive effects of the inhibitor NSC348884 on NPM oligomerization, in comparison with AML cells expressing NPMwt. Recently, we revealed that the localization of NPMmutA is not exclusively cytoplasmic and that a substantial fraction of NPMmutA still resides in the nucleoli [26]. Moreover, we and other authors [26,27] have shown that due to heterooligomer formation, subcellular distribution of NPMmutA changes when the cells are co-transfected with NPMwt. In the present work, we used HEK-293T cell system allowing high amplification of transfected plasmids to investigate the localization of various mutation types. The impact of the endogenous NPM was then analyzed in three cell lines with different ratio of endogenous to exogenous NPM expression. The interaction between various NPM types was further confirmed by co-immunoprecipitation.

Material and methods

This study was conducted in the period 02-11/2016.

Cell culture and chemicals

Cancer cell lines HEK293T (gift from Dr. Š. Němečková, Institute of Hematology and Blood Transfusion, Czech Republic) and NIH 3T3 (gift from Dr. M. Jiroušková, IMG CAS, Czech

Republic) were cultivated in DMEM (Sigma-Aldrich), 10% FCS, 37ÊCand 5% CO_2 atmosphere. Cancer cell line HeLa (gift from Dr. J. MalÂnskÝ, IEM CAS, Czech Republic) was cultivated in RPMI 1640 (Biochrom AG) supplemented with 10% FCS, 37ÊCand 5% CO_2 atmosphere. Peripheral blood mononuclear cells (PBMC) of AML patients were isolated from leukapheretic products using density gradient centrifugation on Histopaque 1077 (Sigma-Aldrich Corporation, USA) at 500 g and 20ÊCfor 25 min. PBMC were resuspended at a density of $5x10^6$ cells/ml in RPMI 1640 medium (10% FCS, 37ÊC,5% CO_2). All patients signed informed consent to the use of their biological material for research purposes in agreement with the Declaration of Helsinki. The Ethics Committee of the Institute of Hematology and Blood Transfusion approved this research at the application of grant No 16-30268A. All samples were tested for presence of C-terminal NPM mutation by PCR and the mutation type was determined by sequencing as described previously [28].

Plasmid construction and cell transfection

As described in detail previously [26], gene for nucleophosmin was amplified from cDNA library (Jurkat cells, Origene) by PCR and inserted to vectors peGFP-C2 and pmRFP1-C2 (originally Clontech) designed for expression of protein chimeras with a fluorescent protein connected to the N-terminus of the target protein by standard methods of molecular cloning. NPM mutants were constructed by PCR using extended primers containing mutated part of exon 12 of the *NPM1* gene and restriction sites (Table 1). After amplification in E. coli, the plasmids with subcloned genes were purified with PureYield Plasmid Miniprep System (Promega) and transfected into adherent cell lines using jetPRIME transfection reagent (Polyplus Transfection) for each experiment. Transfection efficiency was analyzed by flow cytometry (BD Fortessa).

Immunofluorescence

The samples were prepared as described previously [26]. Briefly, cells in suspension were seeded on a coverslip in humidified chamber for 15 min and then fixed with 4% paraformaldehyde (PFA) overnight at 4ÊC.After 10min of permeabilization by 0,5% Triton X-100, the cells were incubated for 1h with a mouse monoclonal anti-NPM primary antibody (clone 3F291, Santa Cruz Biotechnology, 1:100) and for another 1h with the secondary antibody (Alexa-Fluor555-conjugated anti-mouse, Life Technologies, 1:200) and with Hoechst33342 (1μM, Life Technologies). The stained cells were observed under confocal laser scanning microscope FluoView FV1000 (Olympus Corporation).

Live-cell imaging. Subcellular distribution and colocalization of eGFP- or mRFP1-fused variants of nucleophosmin was observed by Olympus FluoView FV1000 confocal microscope (Olympus Corporation). For subcellular distribution statistics, at least 800 cells from three independent experiments were evaluated. Fluorescence images were processed by FluoView software FV10-ASW 3.1.

Cell lysis

Transfected adherent cells were briefly washed with PBS, trypsinized and extensively washed with PBS. The cell pellets were lysed in Laemmli sample buffer, boiled for 5 min, centrifuged at 200.000g/4ÊCfor 4h and the supernatant was stored at -20ÊC.

Immunoprecipitation

GFP-Trap_A system (Chromotek) was used following the manufacturerÂsinstructions. Briefly, transfected adherent cells were resuspended in ice-cold PBS, scrapped from dish and

Table 1. Sequence of extended primers used for construction of the NPM mutants.

Mutation type	Forward primer	Reverse primer
NPM mutA	AAAAAAACTCGAGCATGGAAGATTCGATGGACATAG	AATTAAGGATCCACTATTTTCTTTAAAGAGACTTCCTCCACTGCCAGACAGAGATCTTGAATTAGCCTCTTGGTCAG
NPM mutB	AAAAAAACTCGAGCATGGAAGATTCGATGGACATAG	AATTAAGGATCCACTATTTTCTTTAAAGAGACTTCCTCCACTGCCATGCCAGAGATCTTGAATTAGCCTCTTGGTCAG
NPM mutE	AAAAAAACTCGAGCATGGAAGATTCGATGGACATAG	AATTAAGGATCCACTATTTTCTTTAAAGAGACTTGGGCAAGAGACTGCCAGAGATCTTGAATTAGCCTCTTGGTCAG

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extensively washed with PBS. Then the cell pellet was lysed in the lysis buffer (10mM Tris/Cl pH7.5, 150mM NaCl, 0.5mM EDTA, 0.5% NP-40, protease and phosphatase inhibitors), incubated on ice for 30 min and centrifuged at 20.000g/10min/4ÊC. The lysate was then transferred into the GFP-Trap_A beads and incubated for 1h at 4ÊC. After centrifugation and extensive wash in the diluting buffer (10mM Tris/Cl pH7.5, 150mM NaCl, 0.5mM EDTA), GFP-Trap_A beads were resuspended in SDS-sample buffer, boiled for 5 min and centrifuged at 2.500g/2min/4ÊC. Supernatant was stored at -20ÊCuntil used for SDS-PAGE.

Western blotting

Five microliters of each sample were subjected to SDS-PAGE and transferred into nitrocellulose membrane (Hybond PVDF, Amersham). Mouse monoclonal antibodies against β-actin, GFP and NPM (clone NA24 for wt+mut detection, clone E3 for wt-only detection) were from Santa Cruz Biotechnology. All primary antibodies were used at a dilution 1:100±1:500. Anti-mouse HRP-conjugated secondary antibody was purchased from Thermo Scientific and used at concentrations 1:10,000±1:50,000. ECL Plus Western Blotting Detection System (Amersham) was used for chemiluminescence visualization and evaluation by G-box iChemi XT4 digital imaging device (Syngene Europe).

Statistical analysis

No power calculations were performed. We analyzed all primary AML samples available at the Institute of Hematology and Blood Transfusion during the period 2015±2016 (N = 17). The majority of experiments were performed using cell lines and repeated until the observed differences between groups reached statistical significance. A p-value of 0.05 or lower was pre-set to be indicative of a statistically significant difference between groups compared. In diagrams, arithmetic means of at least three replicates of all experiments were plotted with SD error bars. Significance levels (p values of ANOVA or StudentÂst-test) were determined using InStat Software (GraphPad Software).

Results

Subcellular localization of mutated NPM depends on mutation type

Seventeen PBMC samples from AML patients were screened for the presence of NPM mutation by the PCR and by the immunofluorescence. We detected the NPMwt in 7 (41%) patients, the NPMmutA in 9 (53%) patients and one patient had the mutation type Nm (1108_1109ins CCAG). Blasts with extranuclear NPM localization were found in all samples from the patients with a NPM mutation whereas the localization of NPM was restricted to nucleoli (and partially nuclei) in the samples without mutation (Fig 1).

We transfected HEK-293T cell line with eGFP-labeled variants of NPM and examined the eGFP_NPM subcellular localization under the confocal microscope (Fig 2a). The wild-type NPM and three types of mutated NPM (A, B and E) were analyzed. While the eGFP_NPMwt was detected solely in nucleoli, more than 80% of eGFP_NPMmutA-transfected cells exhibited exclusively cytoplasmic localization of the mutated protein (Fig 2b). A combination of eGFP_NPMmutA signal from the nucleolus with cytoplasmic staining was observed in approximately 15% of the transfected cells. Moreover, the relative fluorescence intensity from the remaining cells, showing eGFP_NPMmutA signal only in the nucleoli (approximately 5% of transfected cells), was weak, indicating low plasmid amplification in these cells. Subcellular distribution of eGFP signal in cells transfected with eGFP_NPMmutB was almost identical as for NPMmutA (Fig 2a). On the contrary, 65% of cells transfected with eGFP_NPMmutE

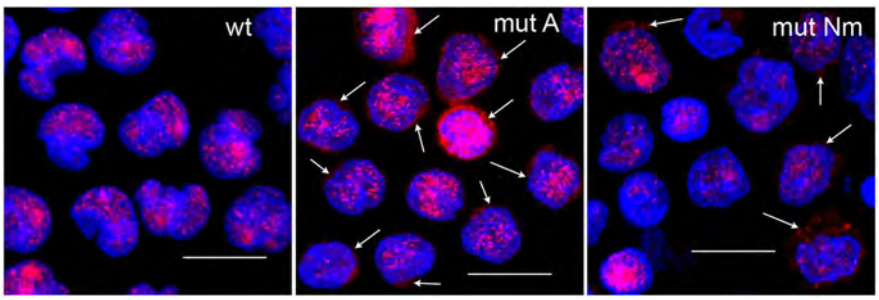

Fig 1. NPM is localized in the cytoplasm of blast from AML patients with NPM mutation. PBMC from AML patients with NPMwt (wt), NPMmutA (mut A) or NPMmutNm (mut Nm) were incubated with anti-NPM (clone 3F291) primary and AlexaFluor555 secondary antibodies (red). The nuclei were visualized with Hoechst 33342 (blue). Arrows indicate the cytoplasmic localization of NPM in AML blasts with NPMmut. The bars represent 10μm.

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displayed eGFP fluorescence from the nucleolus, whether exclusively or partially (i.e. signal was detected from both the cytoplasm and the nucleoli) (Fig 2a and 2b, S1 Table). Identical results were obtained with plasmids containing the red form of the fluorescence protein, mRFP1, instead of eGFP (data not shown). The transfection efficiency measured by flow cytometry was about 45% in all samples and high expression of recombinant fusion proteins was confirmed by immunoblot (Fig 2c, S1 Fig).

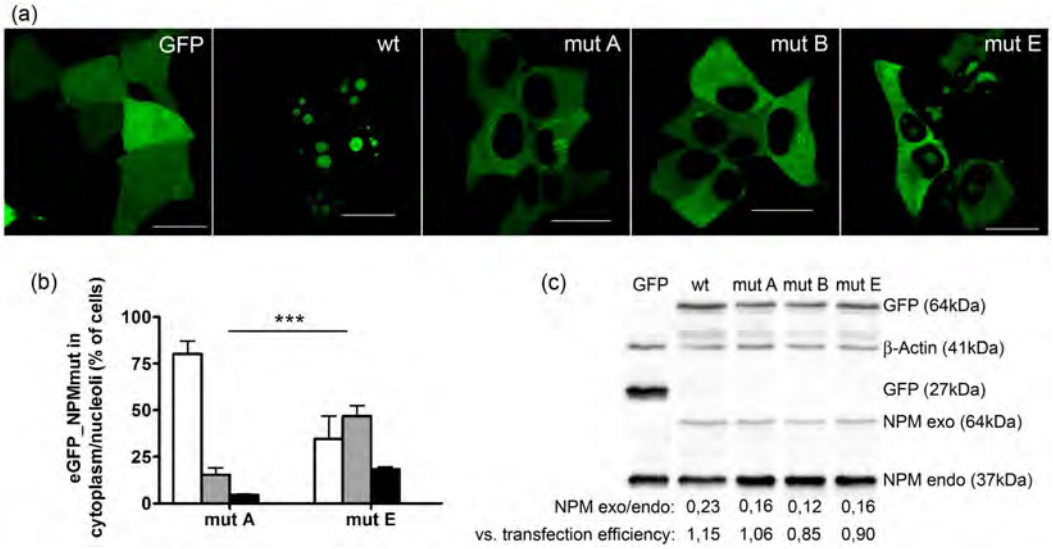

Fig 2. Subcellular distribution of mutated NPM depends on mutation type. (a) eGFP fluorescence from HEK-293T cells transfected with eGFP plasmid (GFP), eGFP_NPMwt (wt), eGFP_NPMmutA (mutA), eGFP_NPMmutB (mutB) or eGFP_NPMmutE (mutE) showing various subcellular distribution of individual NPM variants. The bars represent 20μm. (b) fraction of transfected cells displaying eGFP_NPMmutA (or E) signal only from the cytoplasm (white bars), from the cytoplasm and nucleoli (grey bars) or only from nucleoli (black bars). The error bars in the graph represent ±SD of at least 3 independent experiments. Statistical significance degree of difference between mutA and mutE obtained from two-way ANOVA test was $P < 0.001$ (***). (c) immunoblot of lysates from HEK-293T cells transfected with individual NPM variants. GFP-NPM (exogenous) is detected at 64 kDa, the endogenous NPM at 37 kDa. β-Actin represents the loading control. Densitometric evaluation of NPM exo/endo level and the ratio of NPMexo/endo expression vs the transfection efficiency (20%, 15%, 13,9% resp. 17,8% for wt, mutA, mutB resp. mutE) are indicated for the individual cell lines.

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Interaction between wild-type and mutated NPM

We have previously checked the tug-of-war hypothesis described by Bolli et al [27] suggesting that the localization of both fluorescently labeled wt and mutated NPM forms depends on their mutual ratio. We showed that the abundance of one NPM form caused partial redistribution of its oligomer partner in Hela cells co-transfected with eGFP_NPMmutA and mRFP1_NPMwt [26]. Here we analyzed the distribution of fluorescently labeled NPM variants in HEK-293T cells co-transfected with mRFP1_NPMwt and eGFP_NPMmutA or eGFP_NPMmutE (Fig 3a). For both mutation types, co-transfection with wt caused significant changes in the subcellular distribution: higher fraction of eGFP_NPMmut in the nucleolus as well as a fraction of mRFP1_NPMwt in the cytoplasm was observed in comparison with the distribution in single form-transfected cells (Fig 3b and 3c, S1 and S2 Tables). Our observations prove the fact that the ability of NPM to form oligomers is not disrupted by any type of C-terminal mutation and that heterooligomers between the wild-type and mutated NPM are formed affecting the localization of each other.

Endogenous NPM affects the localization of NPMmut

The subcellular distributions of NPMmutA in both single (NPMmut only) or double (NPMmut+NPMwt) transfected HEK-293T cells markedly differed from the distribution previously observed in HeLa cells [26]. We hypothesize, that the reason for this difference lays in various endogenous expression of NPM in these two cell lines. Karyotype studies of HeLa cells proved a multiplied number of NPM gene copies [29] and a high endogenous NPM expression was thus expected in this cell line. On the other hand, HEK-293T cell line contains the SV40 Large T-antigen, which allows for amplified expression from transfected plasmids containing the SV40 origin of replication. Therefore, we compared the localization and level of NPM protein expression in these two cell lines. In addition, the commonly used mouse NIH-3T3 cell line with standard endogenous NPM expression and unaffected plasmid amplification was analyzed for comparison (Fig 4, S2 Fig, S3 Table). The distribution of eGFP-NPMmutA varied from almost cytoplasmic in HEK-293T to highly nucleolar in HeLa (Fig 4a and 4c, S3 Table). The expression of the endogenous NPM was higher in HeLa compared to the other cell lines (Fig 4b, S2 Fig) and the ratio between the endogenous and the exogenous protein in the transfected cells (Fig 4d, S2 Fig) reflected the high amplification ability of HEK-293T (even with correction for various transfection efficiency in individual cell lines, Table 2).

A good correlation between the fraction of cells with cytoplasmic-only NPMmutA localization and the ratio of exogenous vs. endogenous NPM expression was observed. We suggest that heterodimers are formed not only between the fluorescently labeled NPM forms but also between the recombinant and the endogenous protein. This suggestion was further confirmed by eGFP-precipitation from lysates of transfected cells of HEK-293T and HeLa cell lines using GFP-Trap nanobeads (Fig 5a, S3 Fig). NPM expression was examined by two anti-NPM antibodies. The anti-NPM clone NA24 is directed to recognize the N-terminus of the human NPM and it is thus able to detect the overall NPM, i.e. both the NPMwt and NPMmut. The anti-NPM clone E3 is specific for an epitope at the C-terminus (aa 253±294) of NPMwt and it should hardly recognize the NPMmut. Indeed, whereas the clone NA24 detected GFP-NPM signal from all lysates of transfected cells, the clone E3 generated a signal only from samples transfected with eGFP-NPMwt. On the contrary, both clones equally detected the endogenous NPM in all precipitates containing any type of eGFP_NPM but not in precipitates from untransfected cells. Despite its relatively low expression in HeLa cells, eGFP_NPM effectively co-precipitated the endogenous NPM also in this cell line. Moreover, a higher ratio of the co-precipitated NPMwt vs. the precipitated eGFP_NPM corresponds to higher expression of the

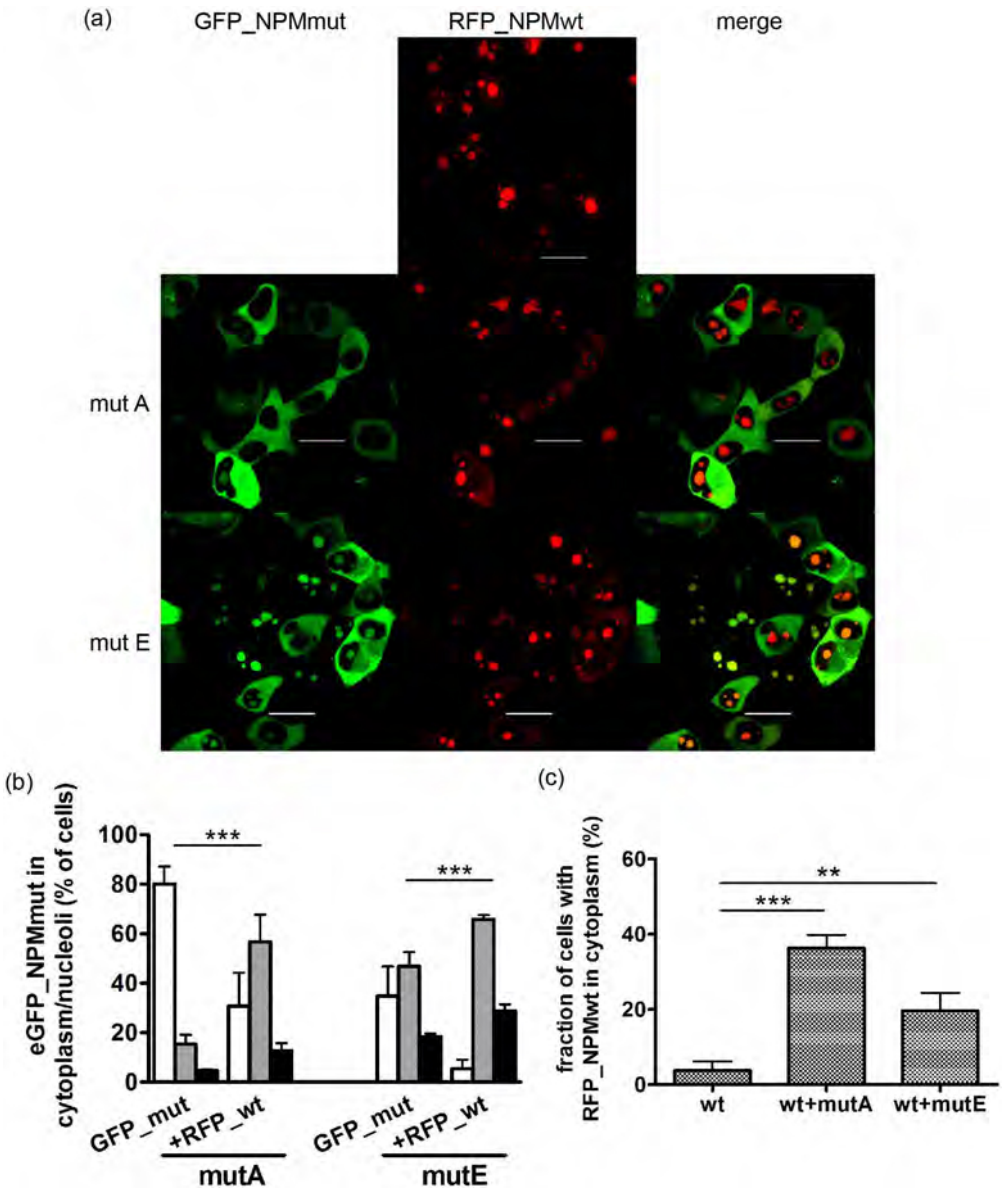

Fig 3. Interaction between wild-type and mutant affects localization of individual forms of NPM. (a) eGFP (green) and mRFP1 (red) fluorescence from HEK-293T cells co-transfected with mRFP1_NPMwt and eGFP_NPMmutA (mutA) or eGFP_NPMmutE (mutE). The bars represent 20 μm. (b) fraction of transfected cells displaying eGFP_NPM signal only from the cytoplasm (white bars), from the cytoplasm and nucleoli (grey bars) or only from nucleoli (black bar). GFP_mut denotes the signal from cells transfected with eGFP_NPMmut only, +RFP_wt denotes eGFP signal from cells co-transfected with eGFP_NPMmut and mRFP1_NPMwt. The error bars in the graph represent ±SD of at least 3 independent experiments. (c) fraction of transfected cells displaying mRFP1_NPMwt signal from the cytoplasm: wtÐcells transfected only with RFP_NPMwt, wt+mutA (or E)±cells co-transfected with RFP_NPMwt and GFP_NPMmutA (or E). The error bars in the graph represent ±SD of 5 independent experiments. Statistical significance degree of difference between the samples: $P < 0.01$ (**), $P < 0.001$ (***).

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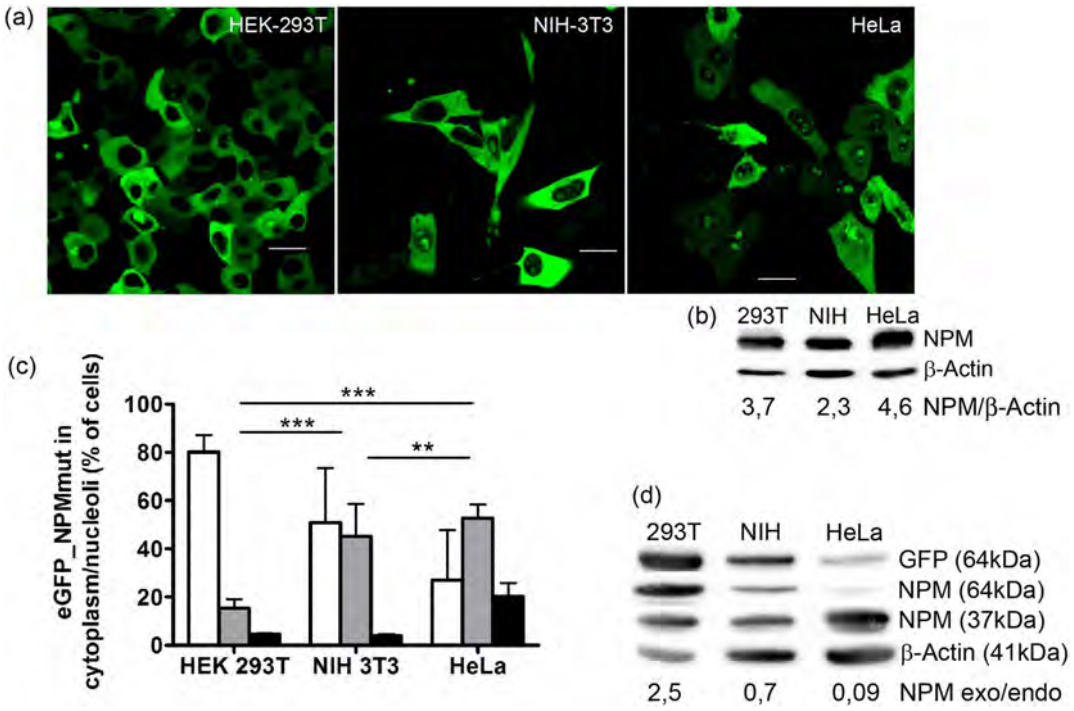

Fig 4. Localization of exogenous NPMmutA depends on endogenous NPM level. (a) eGFP fluorescence from HEK-293T (1), NIH-3T3 (2) or HeLa (3) cells transfected with eGFP_NPMmutA showing its various subcellular distribution in individual cell lines. The bars represent 20μm. (b) immunoblot of lysates from various cell lines indicates different endogenous NPM expression. β-Actin represents the loading control. Densitometric evaluation of NPM/β-Actin ratio is indicated for individual cell lines. (c) fraction of transfected cells displaying eGFP_NPM signal only from the cytoplasm (white bars), from the cytoplasm and nucleoli (grey bars) or only from nucleoli (black bar). The error bars in the graph represent ±SD of at least 3 independent experiments. Statistical significance degree of difference between the samples: $P < 0.01$ (**), $P < 0.001$ (***). (d) immunoblot of lysates from various cell lines transfected with NPMmutA indicates different expression of transfected eGFP_NPM. GFP-NPM (exogenous) is detected at 64 kDa, the endogenous NPM at 37 kDa. β-Actin represents the loading control. Relative ratio of NPM exo/endo expression is indicated for the individual cell lines. Two-fold concentrations of primary and secondary antibodies had to be used to detect exogenous NPM expression in all lines. Therefore, absolute evaluation of the NPM exo/endo expression needs correction for the exo/endo NPM ratio calculated in Fig 2c.

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endogenous NPM in HeLa cells (Fig 5b, S3 Fig). For both cell lines, the amount of co-precipitated NPM was substantially higher in the samples from eGFP_NPMwt-transfected cells than in the samples transfected with eGFP_NPMmut.

Discussion

The significance of specific nucleophosmin mutations in AML has been recognised by the World Health Organization (WHO) which defined the AML with NPM1 mutation as a

Table 2. Transfection efficiency for individual cell lines assessed by flow-cytometry.

	HEK-293T	NIH 3T3	HeLa
transfection ef®ciency (% of cells)	47 ± 13	11 ± 4	19 ± 4
estimated ratio NPM endo:NPM_GFP	1: 1	4: 1	10: 1

mean±SD values from at least 6 samples were calculated. Ratio of NPM forms was estimated from the transfection ef®ciencyand the protein expression levels determined from WB.

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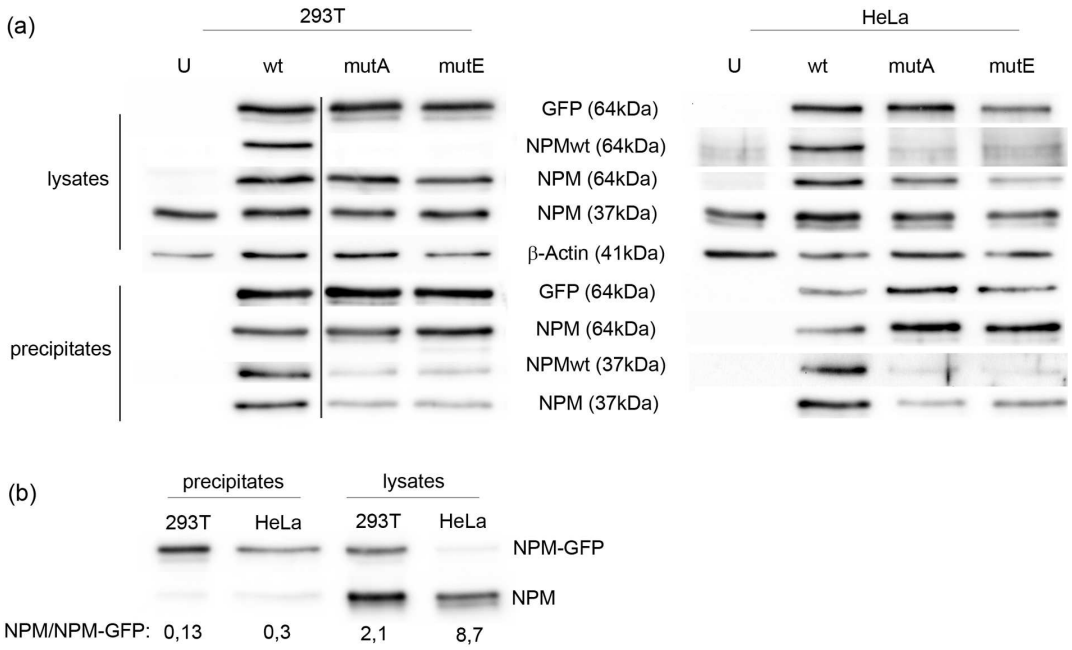

Fig 5. Formation of heterooligomers between eGFP_NPM and endogenous NPM was confirmed by GFP-precipitation. (a) Representative immunoblots of lysates and GFP-precipitates from the cells transfected with individual NPM variants. U: untransfected cells, wt: eGFP_NPMwt, mutA: eGFP_NPMmutA, mutE: eGFP_NPMmutE. Anti-NPM antibody clone NA24 was used to detect the overall NPM expression (i.e. both the NPMwt and NPMmut), the clone E3 was used to detect NPMwt only. GFP-NPM (exogenous) is detected at 64 kDa, the endogenous NPM at 37 kDa. β-Actin represents the loading control. (b) The ratio between endogenous (NPM) and exogenous (NPM-GFP) expression in GFP-precipitates and lysates from cells transfected with eGFP_NPMwt. The membrane from 30 to 100 kDa was incubated with anti-NPM clone NA24.

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distinct entity [24]. The AML with NPM1 mutation without concomitant mutations in other genes is classified into the group with favorable prognosis. However, leukemogenic potential of the mutation as well as the reason for the better outcome are still unclear. The most frequent mutation type (type A) occurs in 75% of patients with NPM mutation, other relatively frequent types B, resp. D are detected in about 9, resp 8% of patients [10,15,30±32]. The mutations A and D differ from each other only in one base of the inserted tetranucleotide, without change in the resulting aminoacid sequence. The type B differs from these types also in a single base, which results in the change in one aminoacid (L289M) in the translated protein [33]. All the proteins resulting from the most frequent mutation types lack both tryptophans W288 and W290 and possess a weak acquired NES motif L-xxx-V-xx-V-x-L. The presence of a NPM mutation in patients with AML was reported to correlate with the cytoplasmic localization of NPM [34]. Consistently with these reports, we observed cytoplasmic NPM localization in all AML samples with NPM mutation (Fig 1). However, the only non-A case in our cohort was of type Nm which is very similar to the type A and the resulting protein differs from NPMmutA in one aminoacid only (L289Q).

The studies investigating the impact of mutation type mostly compared groups of patients with mutation type A versus non-A types. Whereas Koh et al observed worse OS and shorter remission for the non-A group [17], Alpermann et al. reported better survival in patients with non-A mutations [16]. Pastore 2014 [15] found no difference between type A and non-A

groups and, moreover, these authors did not find any difference even in a more detailed discrimination between the types A, B, D and the others (rare). Recently, Alpermann et al. [30] reported that different subtypes of *NPM1* mutation were associated with different profiles with respect to clinical parameters as well as to accompanying molecular markers. Particularly, they revealed that *DNMT3A* mutations worsen the outcome of patients with type A and type D *NPM1* mutations but not with the type B. However, statistics matching the mutations according to their putative subcellular distribution were not performed, probably due to the low frequency of the rare mutations. We suggested previously, that the cytoplasmic localization of NPM is critical for immune therapy prognosis [28]. Therefore, it is important to investigate the difference between the mutation types causing different subcellular localization. In the present work, we examined the localization of three types of NPM mutation (A, B and E) in HEK-293T cell line, in relation to the presence of W288 and the force of the acquired NES motif. We uncovered substantial difference between the localization of the NPMmutA (or B) and the NPMmutE. A high proportion of NPMmutE is retained in the nucleoli in contrast to the mostly cytoplasmic localization of NPMmutA (Fig 2). Interestingly, the mean fluorescence intensity (MFI) determined by flow-cytometry as well as the GFP-NPM level analyzed by immunoblot revealed that the amplification of the plasmids was mostly lower in cells transfected with NPMmut than in NPMwt-transfected cells indicating lower amplification of plasmids containing NPMmut.

In agreement with the experiments described by Bolli et al [27], the localization of each NPMmut type was strongly affected by the co-expression of NPMwt, probably due to hetero-oligomer formation. In cells co-transfected with GFP-NPMmut and RFP-NPMwt, a higher proportion of NPMmut in the nucleoli as well as the NPMwt in the cytoplasm was detected for the both A and E mutation types (Fig 3). The interaction between various NPM forms was further tested in three different cell lines representing various expression systems and pools of the endogenous NPM. A nice correlation of GFP-NPMmut localization with the ratio of exogenous vs. endogenous NPM expression was observed (Fig 4 and Table 2). The interaction between the endogenous and exogenous NPM was further evidenced thanks to GFP-precipitation (Fig 5a). The formation of NPM oligomers or complexes with its interaction partners mediated by its N-terminal domain is largely documented [22,35,36] and the ability of NPM to form oligomers was reported to be retained also in its variants with an altered C-terminus, whether in the fusion protein NPM-ALK [37] or in the protein with specific mutation [38]. Nonetheless, little is known about the potential of the oligomerization domain of the altered protein. In our experiments, a higher proportion of co-precipitated endogenous NPM according to the lowest ratio of exo-/endogenous NPM expression was detected in HeLa cells (Fig 5b). Irrespectively of the cell line, the amount of co-precipitated endogenous NPM was substantially higher in cells transfected with GFP-NPMwt than in cells transfected with any type of NPMmut. This may be partially explained by a higher accessibility of the endogenous NPM for eGFP-NPMwt due to their identical localization. Similar localization should favor the interaction of the endogenous NPM with NPMmutE rather than with NPMmutA, but we did not observe any difference between the levels of co-precipitated endogenous NPM in samples with mutations A and E. Hence, it is possible that the oligomerization potential of NPMmut is lowered when compared to the interaction potential of the wild-type form. This can be supported by the findings of Balusu et al [25] that the cells with NPMmut are more susceptible to a specific inhibitor of NPM oligomerization than the cells with NPMwt and that the NPMmut tends to form dimers rather than oligomers. Recently, it was uncovered that unbalanced allelic expression of mutant alleles is a relatively common occurrence in multiple myeloma patients [39]. The mutant/wild-type allelic ratio for NPM1 has been suggested to have a prognostic value in AML [40]. In

summary, besides the type of the mutation, the oligomerization potential of NPMmut together with the NPMwt/NPMmut ratio considerably affects the subcellular NPM distribution and likely the patientÂsoutcome.

Conclusion

Changes in the intracellular localization contribute very likely to leukemogenicity as well as to the survival advantage which are associated with nucleophosmin mutations in acute myeloid leukemia. Hence, it is important to describe in detail the localization of both the wild-type and the mutated protein in cells with every mutation type. The basic location for the wild-type NPM is in the nucleoli, NPM with mutations A or B reside in the cytoplasm, whereas the form E is found in the cytoplasm, in the nucleus and in the nucleoli. Furthemore, the localization of all these forms is affected by their relative amounts thanks to oligomer formation. Finally, the ability of NPMmut to form oligomers seems to be lowered irrespective of the mutation type.

Supporting information

S1 Fig. Original Western blots with molecular size marker for Fig 2c.
(TIF)

S2 Fig. Original Western blot with molecular size marker for Fig 4b ((a)) and 4d ((b)).
(TIF)

S3 Fig. Original Western blots with molecular size marker for Fig 5a ((a)) and 5b ((b)). Multiple blots were performed from identical samples in one SDS-PAGE run for individual cell lines ((a)).
(TIF)

S1 Table. Subcellular distribution of the mutated NPM type A (mutA) and type E (mutE) in HEK-293T cells transfected with the eGFP_NPMmut (columns mutA/E only) or co-transfected with the eGFP_NPMmut and mRFP1_NPMwt (columns mutA/E + wt). The data from three independent experiments are presented as fractions of cells (% of transfected cells) exhibiting eGFP_NPM signal from the cytoplasm only (C), from the cytoplasm and the nucleoli (C+N) or from nucleoli only (N).
(DOCX)

S2 Table. Fraction of transfected cells displaying mRFP1_NPMwt signal from the cytoplasm. wt onlyÐcells transfected only with RFP_NPMwt, +mutA (or E)±cells co-transfected with RFP_NPMwt and GFP_NPMmutA (or E).
(DOCX)

S3 Table. Subcellular distribution of the mutated NPM type A in HEK-293T, NIH-3T3 or HeLa cells transfected with the GFP_NPMmutA. The data from at least three independent experiments are presented as fractions of cells (% of transfected cells) exhibiting GFP_NPM signal from the cytoplasm only (C), from the cytoplasm and the nucleoli (C+N) or from nucleoli only (N).
(DOCX)

S4 Table. Transfection efficiency of individual cell line transfections with fluorescently labeled forms of NPM.
(DOCX)

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Author Contributions

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Formal analysis: BB MK AH.

Funding acquisition: BB KK.

Investigation: BB MK AH.

Methodology: BB MK AH KK.

Project administration: BB KK.

Resources: BB MK AH KK.

Visualization: BB.

Writing ± original draft: BB MK AH KK.

Writing ± review & editing: BB KK.

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Dodatkové informace k publikaci č. 1: Localization of AML-related nucleophosmin mutant depends on its subtype and is highly affected by its interaction with wild-type NPM1

Tab S1: Subcellular distribution of the mutated NPM type A (mutA) and type E (mutE) in HEK-293T cells transfected with the eGFP_NPMmut (columns mutA/E only) or co-transfected with the eGFP_NPMmut and mRFP1_NPMwt (columns mutA/E + wt). The data from three independent experiments are presented as fractions of cells (% of transfected cells) exhibiting eGFP_NPM signal from the cytoplasm only (C), from the cytoplasm and the nucleoli (C+N) or from nucleoli only (N).

Exper. No.	mutA only			mutA + wt		
	C	C+N	N	C	C+N	N
1	72	23	5	21	70	10
2	84	12	4	25	65	10
3	84	11	5	46	35	19
Exper. No.	mutE only			mutE + wt		
	C	C+N	N	C	C+N	N
1	21	58	20	4	69	27
2	39	43	19	3	63	34
3	44	40	16	10	65	25

Tab S2: Fraction of transfected cells displaying mRFP1_NPMwt signal from the cytoplasm: wt only - cells transfected only with RFP_NPMwt, +mutA (or E) – cells co-transfected with RFP_NPMwt and GFP_NPMmutA (or E).

Exper. No.	% of cells with NPMwt in cytoplasm		
	wt only	+mutA	+mutE
1	1	39	21
2	4	40	18
3	6	32	23
4		37	24
5		33	12

Tab S3: Subcellular distribution of the mutated NPM type A in HEK-293T, NIH-3T3 or HeLa cells transfected with the GFP_NPMmutA. The data from at least three independent experiments are presented as fractions of cells (% of transfected cells) exhibiting GFP_NPM signal from the cytoplasm only (C), from the cytoplasm and the nucleoli (C+N) or from nucleoli only (N).

Exper. No.	HEK 293T			NIH 3T3			HeLa		
	C	C+N	N	C	C+N	N	C	C+N	N
1	72	23	5	75	21	4	27	56	17
2	84	12	4	47	48	5	18	61	21
3	84	11	5	31	67	2	56	36	8
4							8	57	35

Tab S4: Transfection efficiency of individual cell line transfections with fluorescently labeled forms of NPM.

	HEK	NIH	HeLa
	61	13,2	24
	41,9	12,4	20,7
	42,5	6,4	15,6
	29,3	6,5	21,1
	56,8	11,5	19,6
	48,7	10,4	12,1
	44,1	19	15
	63,7		20,4
	57,6		22,2
	55,5		22,2
	66,1		18,5
	49,9		16,8
	54,1		
	48,2		
	38,5		
	36,6		
	30		
	31		
	47		
	61		
	34,8		
	33,9		
	23,8		
	28,4		
	20,1		
	57,7		
	49,8		
	66,2		
	49,6		
	64,9		
	43,8		
	70		
	55		
	45		
Mean	47	11	19
SD	13	4	4

Fig S1: Original Western blots with molecular size marker for Fig. 2c.

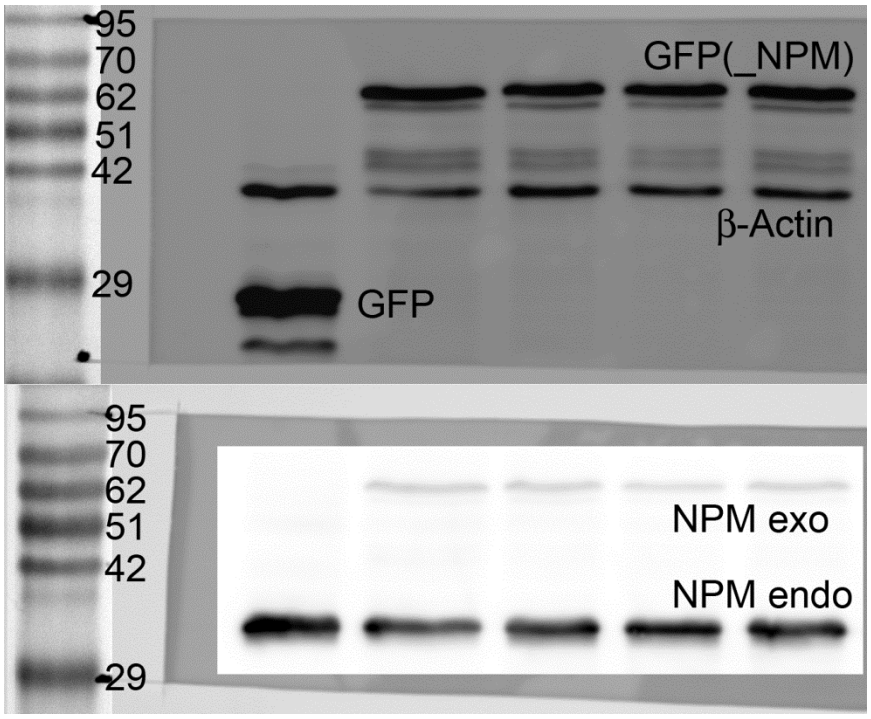


Fig S2A: Original Western blot with molecular size marker for Fig 4b.

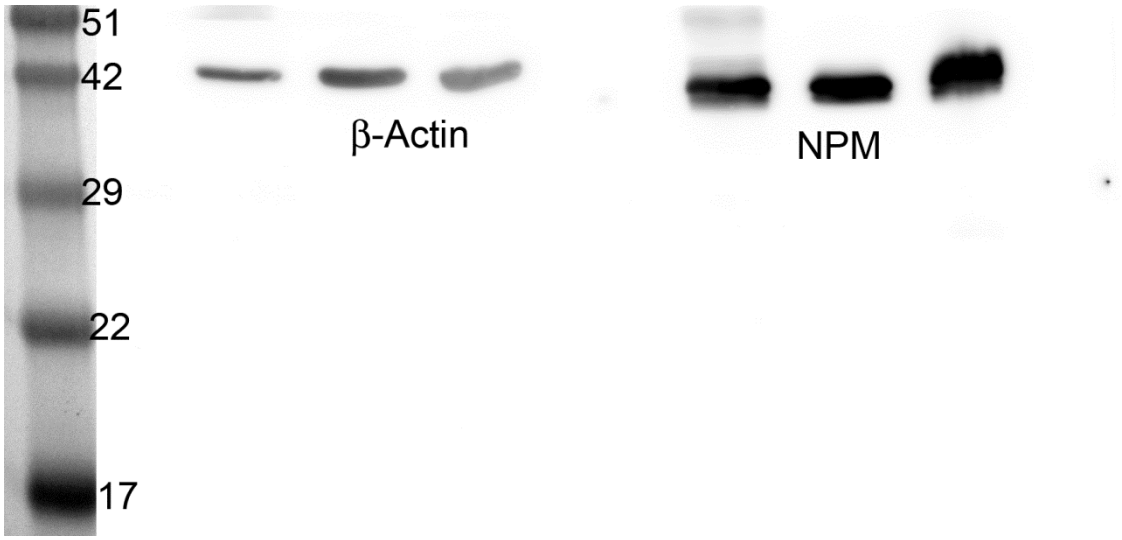


Fig S2B: Original Western blot with molecular size marker for Fig 4d.

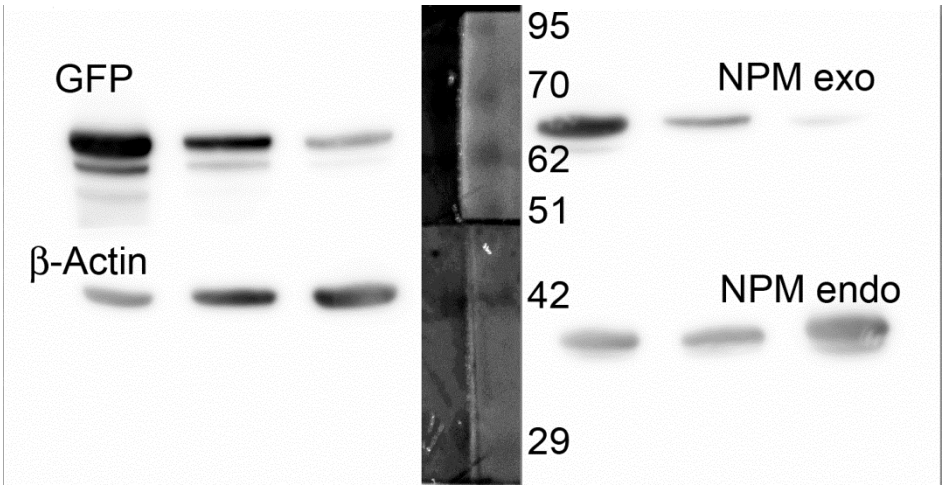


Fig S3A: Original Western blots with molecular size marker for Fig 5a. Multiple blots were performed from identical samples in one SDS-PAGE run for individual cell lines.

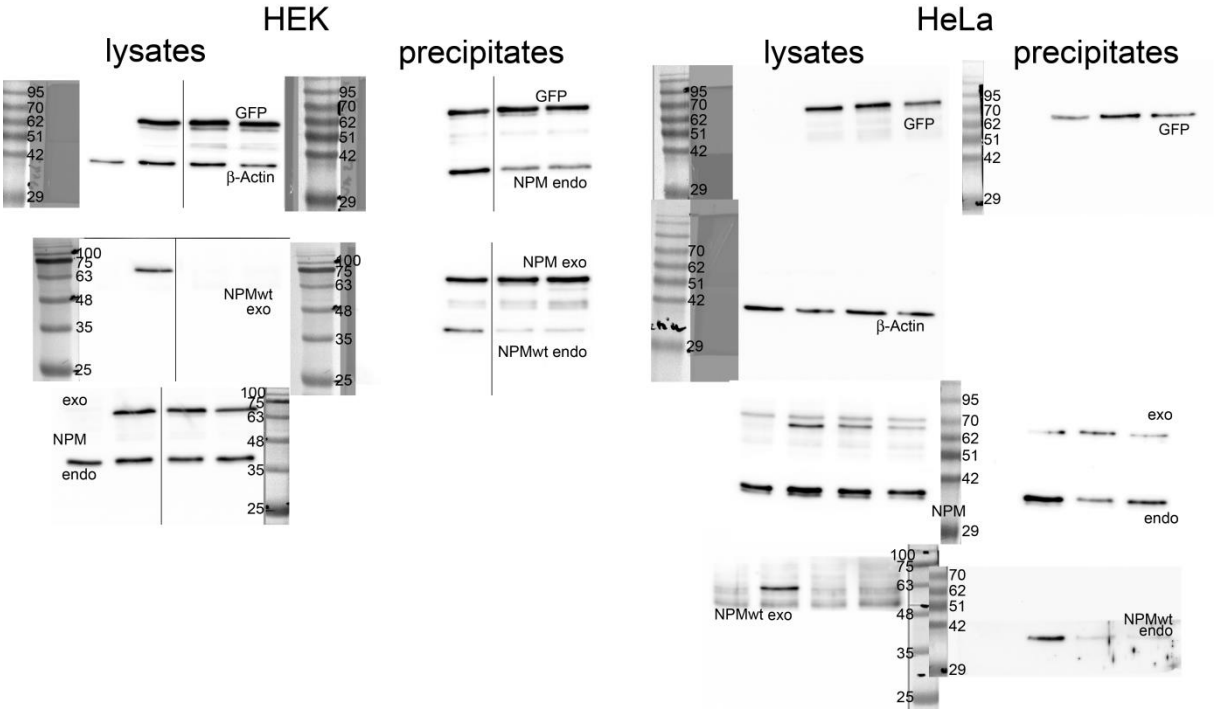
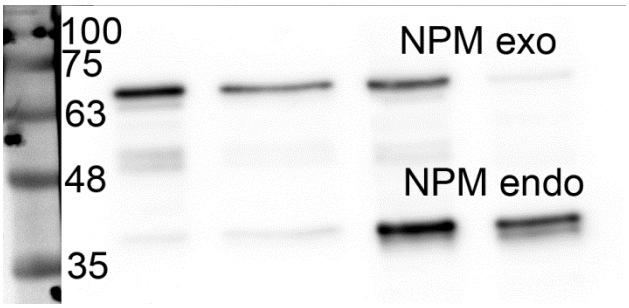


Fig S3B: Original Western blot with molecular size marker for Fig 5b.



8.2 Příloha č. 2: Monitoring of nucleophosmin oligomerization in live cells

Methods and Applications in Fluorescence

PAPER

Monitoring of nucleophosmin oligomerization in live cells

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Abstract

Oligomerization plays a crucial role in the function of nucleophosmin (NPM), an abundant nucleolar phosphoprotein. Two dual-color methods based on modern fluorescence confocal microscopy are applied for tracking NPM aggregates in live cells: cross-correlation Number and Brightness analysis (ccN&B) combined with pulsed interleaved excitation (PIE) and fluorescence-lifetime imaging microscopy (FLIM) utilizing resonance energy transfer (FRET). HEK-293T cells were transfected with mixture of plasmids designed for tagging with fluorescent proteins so that the cells express mixed population of NPM labeled either with eGFP or mRFP1. We observe joint oligomers formed from the fluorescently labeled NPM. Having validated the *in vivo* methods, we study an effect of substitutions in cysteine 21 (Cys21) of the NPM N-terminus on the oligomerization to demonstrate applicability of the methods. Inhibitory effect of mutations of the Cys21 to nonpolar Ala or to aromatic Phe on the oligomerization was reported in literature using *in vitro* semi-native electrophoresis. However, we do not detect any break-up of the joint NPM oligomers due to the Cys21 mutations in live cells. *In vivo* microscopy observations are supported by an *in vitro* method, the GFP-Trap immunoprecipitation assay. Our results therefore show importance of utilizing several methods for detection of biologically relevant protein aggregates. *In vivo* monitoring of the NPM oligomerization, a potential cancer therapy target, by the presented methods offers a new way to monitor effects of drugs that are tested as NPM oligomerization inhibitors directly in live cells.

Abbreviations

AML	acute myeloid leukemia
ccN&B	cross-correlation N&B analysis
FFS	fluorescence fluctuation spectroscopy
FLIM	fluorescence lifetime imaging microscopy
FP	fluorescent proteins
FRET	Förster (fluorescence) resonance energy transfer
NCL	nucleolin
NPM	nucleophosmin
PIE	pulsed interleaved excitation

1. Introduction

Nucleophosmin (NPM) is an abundant nucleolar phosphoprotein with strong expression in proliferating cells [1–3]. NPM, similarly to another abundant nucleolar phosphoprotein nucleolin (NCL), is thought to function as a hub protein. NPM is thus capable of interacting with other nucleolar assembly proteins [4].

They are involved in various types of cancer. NPM is frequently overexpressed in solid tumors [5, 6]. NPM is upregulated in relapsed/refractory acute leukemia as shown by proteomic analysis [7]. Moreover, NPM is mutated or fused to other proteins in hematological disorders [6, 8]. The *NPM*1 gene mutation resulting in changes of the NPM C-terminus is the most frequent genetic aberration in the acute myeloid leukemia (AML) [9]. These changes cause relocation of the C-terminus-mutated NPM (NPMc+) from the nucleolus into the cytoplasm [10, 11]. In the cells expressing the NPMc+, NPM-interacting tumor supressors such as p53 or ARF are readily dislocated to the cytoplasm bound to NPMc+, which interferes with their proper regulative and anti-malignant action [12–14].

Oligomerization state of the NPM is critical for its function and dynamics [15–17]. NPM, a member of nucleoplasmin family of proteins [18, 19], forms pentamers in a ring-like configuration [20, 21]. Current models of the pentameric NPM arrangement are based on x-ray crystallography of the core oligomerization domain without protein C-terminus [22]. NPM N-terminus domain is reported to be responsible for the oligomerization [23, 24]. Cysteines Cys21 and Cys104 present in the N-terminus were identified to play important role in the NPM oligomerization [16, 25]. Formation of the NPM pentamers was inhibited by mutation of Cys21 to Ala or to aromatic hydrophobic residues (Phe or Tyr). The C21F mutant expressed in MCF7 cells did not oligomerize with endogenous wild-type (WT) NPM [16]. Conserved tyrosine 67 in β-hairpin was also found to affect the NPM oligomerization in dominant-negative fashion [26]. On the other hand, the NPMc+ keeps its ability to form oligomers [25, 27].

The NPM oligomerization, specifically the N-terminus of NPM responsible for the oligomerization, is being considered a promising target for the cancer therapy [28, 29]. Though only the Phe and not the Tyr substitution of Cys21 drastically inhibited chaperone activity of NPM [16], it is supposed that the oligomerization of NPM is linked to its molecular chaperon activity [1]. Furthermore, it is supposed that the ability of NPM to interact with the tumor suppressors depends also on its oligomerization status [6, 30, 31]. Targeting the NPM interaction ability via modulation of its oligomerization should help to relocalize the tumor suppressors back to the nucleus. Their pro-apoptotic activity should be restored there, possibly in cooperation with WT NPM resting in the nucleus. The small molecule inhibitor NSC348884 was identified to interfere with the oligomerization thereby affecting differentiation and apoptosis in leukemic cells [15, 32, 33]. Treatment with cytotoxic agents such as actinomycin D did not affect the NPM oligomerization in HeLa cells [34]. The oligomerization was changed during apoptosis induced by tumor necrosis factor [35].

The oligomerization of NPM has been extensively studied *in vitro*. Various methods, such as PAGE [16, 31], native electrophoresis [17, 34], sedimentation in saccharose gradient [1, 34, 36], co-immunoprecipitation with epitope tags [27, 36, 37], deuterium exchange mass spectrometry (DXMS) [26], size exclusion chromatography [1] and split synthetic *Renilla* luciferase protein fragment-assisted complementation (SRL-PFAC) [38], were used. However, our ability to understand oligomerization processes in cells is still limited [39]. Data on the NPM oligomerization *in vivo* are mostly missing. Fusing NPM with colored variants of fluorescent proteins (FP) enables application of fluorescence-based methods to monitor protein-protein interactions *in vivo*. Classical one-color Number and Brightness (N&B) is a fluorescence fluctuation spectroscopy (FFS) method [40], in which a number of fluorescing molecules and their brightness are statistically evaluated for each pixel along series of images. This enables to estimate the aggregation state of the fluorescing species [41, 42]. N&B method was reported to provide reliable information on molecular aggregation *in vivo*, including additional spatial information contained in 2D brightness maps [41]. Standard transfection protocols provide relatively high expression levels of fluorescently labeled proteins which is ideal for imaging but unfavorable for one-color N&B, because fluctuations of the fluorescence intensity of individual molecules are difficult to resolve against a relatively high signal. Based on a simultaneous two color detection, dual-color FFS techniques, such as fluorescence cross-correlation spectroscopy (FCCS) [43, 44] or cross-correlation Number and Brightness analysis (ccN&B) [40, 45], offer convenient tools to detect protein aggregates in live cells. Having cloned plasmids for expression of NPM fused with eGFP and mRFP1 [46], we apply the ccN&B [47] to monitor joint aggregates formed by NPM tagged with both FP variants. The ccN&B is combined with a pulsed interleaved excitation (PIE) [48, 49], which enhances the method sensitivity by suppressing spectral cross-talk caused by bleeding of eGFP fluorescence to mRFP1 detection channel. We validate the method in live cells by using appropriate controls.

The FFS-based methods can be complemented by the Förster resonance energy transfer (FRET) reporting on proximity of the FP-tagged proteins. In FRET, transfer of excitation energy from donor, e.g. eGFP, to acceptor, e.g. mRFP1, depends strongly on their distance in the nm range. As FRET also manifests in shortening of the donor fluorescence lifetime [50, 51], proximity of the tagged proteins can be effectively monitored by fluorescence lifetime imaging microscopy (FLIM). We observe shortening of eGFP fluorescence lifetime in a mixture of eGFP- and mRFP-tagged NPM and we attribute this shortening to FRET by acceptor photobleaching. The FLIM-FRET can therefore instantly monitor the co-localization of the

FP-tagged NPM in aggregates formed in live cells [50–53]. In agreement with the ccN&B method, FLIM-FRET experiments verify our observations made when studying NPM substituted in the Cys21. Finally, we confirm the unexpected stability of the Cys21-mutated NPM aggregates also with an *in vitro* method, co-immunoprecipitation GFP-Trap assay.

2. Material and methods

2.1. Cell cultivations

Adherent cell line HEK-293T (a gift from Š Němečková's lab, Institute of Hematology and Blood Transfusion) was cultured under standard cultivation conditions in DMEM (SIGMA) supplemented with 10% FBS (SIGMA), 37 °C and 5% CO_2 atmosphere.

2.2. Flow cytometry

Co-transfection efficiency was tested by flow cytometry (LSR Fortessa, BD Biosciences). Cells were detached from cultivation dish with Trypsin (SIGMA), dissolved in DMEM medium to block Trypsin, span down and then resuspended in PBS. Fluorescence originating from eGFP and mRFP1 tags was registered in FITC and PE channels, respectively. Green and red -positive events were assessed for at least 20 000 cells for each sample.

2.3. Co-immunoprecipitation GFP-Trap assay

Transfected cells expressing fluorescent proteins were processed after 40h-incubation. GFP-Trap_A system (Chromotek) was used following the manufacturer's instructions as described in [27]. Briefly, eGFP expressing adherent cells were scrapped from dish in ice-cold PBS and extensively washed with PBS. The cell pellet was lysed in the lysis buffer (10 mM Tris/Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, protease and phosphatase inhibitors) on ice for 30 min and centrifuged at 20.000 g/10 min/4 °C. The lysate was applied on the GFP-Trap_A beads and rotated for 1 h at 4 °C. Then the samples were centrifuged and extensively washed in the diluting buffer (10 mM Tris/Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA) to be resuspended in 2xSDS-sample buffer (100 mM Tris pH 6.8, 4% SDS, 200 mM DTT, 20% glycerol), boiled for 10 min and centrifuged at 2.500 g/2 min/4 °C. Supernatant was stored at −20 °C until used for SDS-PAGE. Western blotting was carried out according to [27]. Mouse monoclonal antibodies against GFP and NPM (clone 3F291) were from Santa Cruz Biotechnology and they were used at a dilution 1:500. Anti-mouse HRP-conjugated secondary antibody was purchased from Thermo Scientific and used at concentrations 1:50 000. Blots were visualized with ECL Plus Western Blotting Detection System (GE Healthcare) and evaluated by G-box iChemi XT4 digital imaging device (Syngene Europe).

2.4. Plasmid construction and cell transfection

Plasmids for expression of fluorescently tagged proteins were constructed by standard molecular cloning techniques (cutting with restriction enzymes (Thermo Scientific) and ligation (NEB)). Plasmids for NPM and NCL expression were prepared as described in [46]. Fragments, PCR-amplified from cDNA library (Jurkat cells, Origene), were subcloned to vectors peGFP-C2 or pmRFP1-C2 (originally Clontech) using XhoI and BamHI unique restriction sites. Mutations of Cys21 were introduced to the sequence of WT NPM using merged primers containing appropriate mutation and XhoI restriction site (table 1). Following PCR amplification, the resulting fragments were subcloned into the peGFP-C2 and the pmRFP1-C2 as in the case of the WT NPM form.

The constructed plasmids were amplified in *E. coli* and purified with PureYield Plasmid Miniprep System (Promega). For transfection, cells were seeded to 1×10^5/ml cell density 24 h prior transfection. Expression plasmids were transfected into HEK-293T cells using jetPrime transfection reagent (Polyplus transfection) according to manufacturer's protocol. Growth medium was replaced 4 h after transfection and cells were then grown 40 h prior analysis.

The NPM constructs and their combinations which were prepared or used in this study are summarized in table 2. In addition, combination of NCL constructs is mentioned.

2.5. Live cell imaging

The cells were grown on glass bottom Petri dish (Cellvis). Fluorescence experiments were carried out at a room temperature after sealing the Petri dish with parafilm to prevent CO_2 leakage. One Petri dish sample was measured typically within 1 h. The subcellular distribution and colocalization of eGFP- or mRFP1-fused NPM variants were observed under a confocal laser scanning microscope FluoView FV1000 (Olympus Corporation) as in [46]. Fluorescence images were processed by FluoView FV10-ASW 3.1 software and by ImageJ-FiJi.

2.6. PIE-ccN&B data acquisition and analysis

PIE-ccN&B cross-correlation experiments utilized dual-color laser excitation set-up with a pulsed 'blue' source (Picoquant, LDH-DC-470, 470 nm) and a 'green' continuous wave source (Uniphase, He-Ne laser, 543 nm). The excitation beam of both wavelengths was guided into the sample via 488/543/633 dichroic mirror (Olympus) and water immersion objective (Olympus 60x, NA 1.2). The emitted fluorescence was then guided through a pinhole (120 μm), coupled into the multimode optical fiber and at its exit it was split into two detection channels separated by a 535 DCXC dichroic mirror (Chroma filters). The 'blue' and 'green' emission channels were equipped with the bandpass filters transmitting wavelengths of

Table 1. Primers used in plasmid construction for PCR amplification.

G_NPM forward	aaaaaactcgagcatggaagattcgatggacatg
G_NPM(C21A) forward	aattaactcgagcatggaagattcgatggacatggacatgagccccctgaggccccagaactatcttttcggtgctgaactaaaggc
G_NPM(C21F) forward	aattaactcgagcatggaagattcgatggacatggacatgagccccctgaggccccagaactatcttttcggtttcgaactaaaggc
G_NPM reverse	aaaaaaggatcccttaaagagacttcctccactgc

Table 2. Coding of constructs and their combinations (symbol '/' denotes mixture of constructs).

R_NPM_G	positive control, bi-colored NPM with mRFP1 and eGFP attached on N- and C-terminus, respectively
G_NPM/R_NPM	two color NPM (mixture of NPM tagged with eGFP or mRFP1, both from the N-terminus)
G_NPM	NPM tagged with eGFP from the N-terminus
G_NPM/R	negative control (mixture of NPM tagged with eGFP from the N-terminus and free mRFP1)
G_NCL/R_NCL	two color NCL (mixture of NCL tagged with eGFP or mRFP1, both from the N-terminus)
G_NPM-C21A	NPM C21A mutant tagged with eGFP from the N-terminus
G_NPM-C21F	NPM C21F mutant tagged with eGFP from the N-terminus
G_NPM-C21A/R_NPM-C21A	two color C21A NPM mutant (mixture of G_NPM-C21A tagged with eGFP or mRFP1, both from the N-terminus)
G_NPM-C21F/R_NPM-C21F	two color C21F NPM mutant (mixture of G_NPM-C21F tagged with eGFP or mRFP1, both from the N-terminus)

515 ± 15 nm and 605 ± 20 nm (Chroma filters), respectively. Signal was detected with two single photon counting modules (tau-SPAD, Picoquant) and the arrival times were recorded with multichannel event timer with TCSPC option (Hydraharp 400, Germany) synchronized with the Galvo scan unit (Fluoview 1000, Olympus). This arrangement enabled us to operate the experiments in the PIE mode adjusted for the combination of pulsed and continuous wave laser. Specifically, the 'blue' pulsed laser was operated at 10 MHz repetition rate (i.e. approximately 100 ns temporal spacing between the adjacent pulses). As the lifetime of the eGFP fluorophore is about 2.5 ns, the mRFP1 emission excited exclusively by the continuous wave 'green' source can be extracted by means of the time-gating (i.e. the signal coming in last 50 ns within the TCSPC histogram was taken), which suppressed completely the undesired cross-talk artifact. For the PIE-ccN&B cross-correlation experiments, we adapted the protocol originally developed for the Raster image correlation spectroscopy (RICS) [54]. For each experiment, 100 frames consisting of 84 × 82 pixels were collected. The scanner was operated at the speed of 100 μs/pixel with a step of 50 nm. The image stacks were taken only from the nucleoplasm which displayed relatively uniform fluorescence intensity.

The values of eGFP and mRFP1-brightness (B_1 and B_2, respectively) and cross-brightness (B_{cc}) can be calculated for each pixel within the recorded set of images as described in [55, 56]. In brief, prior to the brightness calculation the images were corrected for bleaching by a detrend procedure accounting for the Poissonian distribution of the recorded signal. The cross-variance σ_{cc} of the fluorescence signal, and subsequently the cross-brightness B_{cc}, were then evaluated for the eGFP and mRFP1 channels as follows:

$$\sigma_{cc}^2 = \frac{\sum(I_1 - \langle I_1\rangle)((I_2 - \langle I_2\rangle)}{K} \quad (1)$$

$$B_{cc} = \frac{\sigma_{cc}^2}{\sqrt{\langle I_1\rangle \langle I_2\rangle}}, \quad (2)$$

where I_1 and I_2 stand for the fluorescence intensity in the eGFP and mRFP1 channel, respectively, $\langle I_1\rangle$ and $\langle I_2\rangle$ stand for their means and K is the number of evaluated frames.

2.7. FLIM—data acquisition and analysis

FLIM experiments were carried out on an inverted IX83 microscope with FV1200 confocal scanner (Olympus). The microscope was equipped with a FLIM add-on comprising picosecond semiconductor lasers, fibre-coupled GaAsP hybrid detectors, and TimeHarp 260PICO TCSPC detection electronics (all PicoQuant, Berlin, Germany). The instrument was complemented with frequency-doubled OPO (Chameleon compact), laser (Coherent, Santa Clara, USA) for tunable VIS excitation used in photobleaching experiments. Specifically, cellular FLIM experiments were performed with a UPLSAPO 60x NA 1.2 water immersion objective (Olympus). eGFP fluorescence was excited at 485 nm by a LDH-DC-485 laser head (PicoQuant), emission decays were collected on the pixel-by-pixel basis in the epi-fluorescence mode using combination of 560 nm short-pass dichroic and Semrock 520/35 bandpass filters in the descanned detection path. To avoid pile-up, the data collection rate was kept below 5% of the laser repetition rate. FLIM data were analyzed by the SymPhoTime64 software (PicoQuant). The lifetime images were generated by a robust 'fast-FLIM' approach when mean pixel lifetimes were calculated by a method of moments [57]. Specifically, the mean lifetime τ_{av} for each pixel was determined as the difference between the barycentre of the fluorescence decay recorded at one position of the scanner and the offset t_{offset} at the

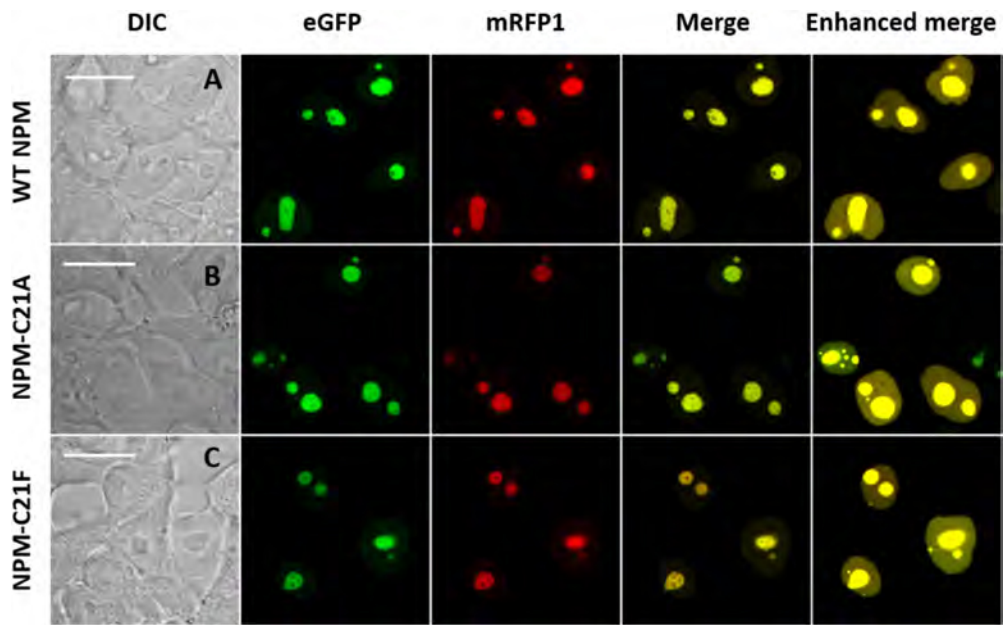

Figure 1. Cellular localization of NPM variants tagged by fluorescent proteins; Localization of G_NPM and R_NPM corresponds to the natural nucleolar localization of WT NPM (row **A**). C21A and C21F NPM mutants exhibit a similar nuclear localization (row **B** and row **C,** respectively). All NPM forms, regardless of the fluorescent protein variant used for their labeling, are located mainly in the nucleolus. Co-localization of the colored forms is shown in the merge images. Minor nucleoplasmic localization of NPM is shown in the enhanced merge images acquired with augmented excitation. The Cys21 mutations do not significantly affect nucleoplasmic NPM levels. Bars represent 20 μm.

steepest uprise of the decay curve:

$$\tau_{av} = \frac{\sum I_i t_i}{\sum I_i} - t_{offset}, \quad (3)$$

where I_i stands for the intensity recorded in time t_i. Least-squares reconvolution was applied for accurate analysis of cumulative decays from larger ROIs (cell, nucleus, nucleolus). Fluorescence of eGFP was assumed to decay bi-exponentially [53, 58]:

$$I(t) = a_1 \cdot e^{-t/\tau_1} + a_2 \cdot e^{-t/\tau_2}, \quad (4)$$

where τ_i are lifetime components and a_i stand for corresponding amplitudes. The intensity-weighted mean fluorescence lifetime was calculated as:

$$\tau_{mean} = \sum f_i \cdot \tau_i, \quad f_i = a_i \tau_i / \sum a_i \tau_i, \quad (5)$$

where f_i are intensity fractions of the *i*th lifetime component. Correlation plots were done in Matlab.

2.7.1. Statistical analysis

Statistical analysis was performed using the GraphPad Prism software. Student's t test was performed for statistical analysis, p values of $p < 0.05$ were deemed statistically significant. Asterisks are used to denote statistical significance: *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

3. Results

3.1. Expression of dual-color labeled NPM variants in HEK-293T cells

Methods for the NPM oligomerization monitoring based on the cross-correlation FFS (ccFFS) require dual-color labeling. We transfected HEK-293T cells with 1:1 mixtures of plasmids (table 2), as in [46], to ensure simultaneous expression of eGFP- and mRFP1-tagged NPM inside the cell. Typical confocal images of cells expressing NPM variants labeled with both colors are shown in figure 1.

Good controls are an essential prerequisite for reliable identification of interacting proteins by means of the ccFFS methods. In cross-correlation experiments based in the PIE-ccN&B, we used cells expressing R_NPM_G (table 2) carrying both eGFP and mRFP1 on the same protein as a positive control and cells expressing combination of G_NPM (table 2) with free mRFP1 as a negative control. Confocal images of these controls are presented in figure 2. The eGFP and mRFP1 proteins bound within the R_NPM_G should drift together in the cell nucleus. R_NPM_G was found to be localized both in the nucleus and the nucleolus (figure 2(A)), with weaker preference for the nucleolus compared to the WT NPM (figure 1(A)). The common movement should result in positive value of cross-brightness B_{cc} obtained by the ccN&B (see equation (2)). For the negative control we used the G_NPM complemented with free mRFP1 expressed

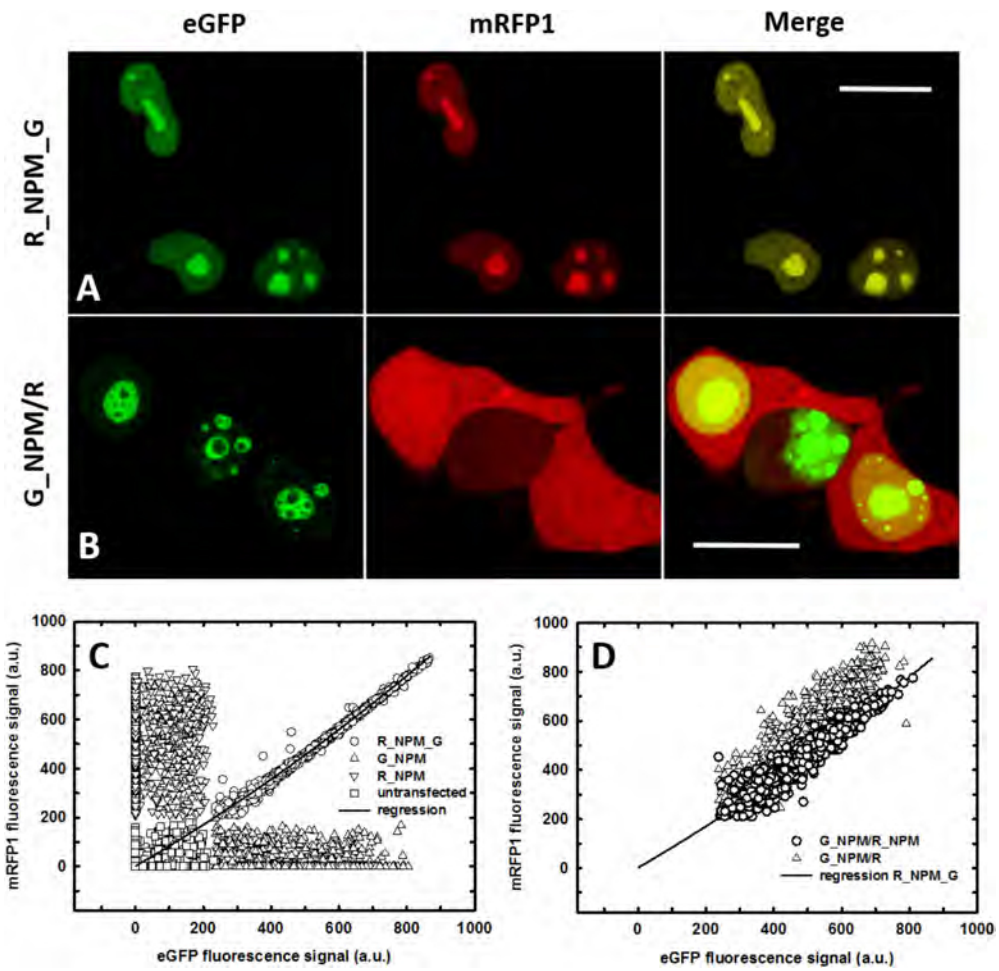

Figure 2. Dual-color labeling of cells expressing controls for the PIE-ccN&B and labeling of cells expressing the G_NPM/R_NPM mix are tested by microscopy imaging and by flow cytometry; (row **A**)—Cells expressing the R_NPM_G construct are used as a positive control. Presence of the bi-colored NPM in nucleoplasm is stronger compared to the WT NPM in figure 1. (row **B**)—Cells expressing G_NPM/R mix serve as a negative control. Free mRFP1 spreads throughout the cell. Co-localization with G_NPM in the nucleoplasm is displayed in the merge image where the G_NPM nucleoplasm localization is enhanced by augmented excitation. Bars represent 20 μm. In **C** and **D**, HEK-293T cells co-transfection by red and green fluorescent tags was evaluated by flow cytometry with simultaneous detection of green and red fluorescence. (**C**)—Flow cytometry data of the cells expressing the R_NPM_G construct exhibit diagonal distribution (○) which shows simultaneous labeling of the cells with both fluorescent tags. Data of cells expressing single tags only, i.e. G_NPM (△) and R_NPM (▽), and background signal of unlabeled cells (□) are included to illustrate thresholding of the dual-color events. The R_NPM_G events are fitted with a polynomial curve. (**D**)—Events registered for the cells expressing the G_NPM/R_NPM mix (○) are distributed along the polynomial curve fitted to the R_NPM_G events in **C**. Dual labeling was confirmed also for the NPM_G/R negative control (△).

from the empty cloning vector. To compensate for higher expression of mRFP1 compared to G_NPM, either cells with lower mRFP1 signal were selected for the experiment or cells were transfected with lower amount of the mRFP1 plasmid.

The dual labeling of cells was verified by flow cytometry (figures 2(C), (D)). Cells expressing the R_NPM_G served as a reference. Since each fused protein carries pair of the flurescent proteins each cell expressing the bi-colored NPM should contain the same amount of eGFP and mRFP1. Indeed, events detected for cells expressing the R_NPM_G lay approximately on a diagonal (figure 2(C)). Additionally, events registered for cells expressing G_NPM/R_NPM mixture (1:1) exhibit broad intensity distribution along a curve fitted to the R_NPM_G events (figure 2(D)). This confirms simultaneous expression of both eGFP- and mRFP1-tagged NPM which is a good prerequisite for successful application of the ccN&B in detection of bi-colored aggregates.

Inhibition of the NPM oligomerization by mutation of Cys21 to Ala or Phe was already reported [16, 25]. To have a possibility to test NPM oligomerization monitoring by the PIE-ccN&B, NPM gene in plasmids for FP-tagged expression was mutated by molecular cloning to provide the Cys21-mutated NPM variants. HEK-293T cells were transfected with 1:1 mixtures of the plasmids to express the WT NPM

and the NPM mutants fused either with eGFP or mRFP1. Localization of the two color NPM mutants in cells was verified by the confocal fluorescence microscope compared to WT NPM (figure 1). The WT NPM localizes preferentially to the nucleolus as expected (figure 1(A)). Compromising the oligomerization by the substitutions in the Cys21 should result in delocalization of NPM to the nucleoplasm [31]. However, we observed that all the NPM variants labeled with eGFP or mRFP1 are localized mainly in nucleoli (figures 1(B), (C)). Hence, contrary to the expectations, the Cys21 mutants do not show any signs of delocalization from the nucleolus to the nucleoplasm. The tagged mutants are matching the tagged WT NPM in their localization and nucleoplasma levels. Therefore, the inhibition effect of the Cys21 substitutions on the NPM oligomerization is challenged by these microscopy observations in live cells.

3.2. PIE-ccN&B -based monitoring of the NPM oligomerization

Provided that joint aggregates consisting of both eGFP- and mRFP1-labeled NPM are formed in transfected cells, the aggregates emit green and red fluorescence simultaneously. Therefore, green and red fluorescence signals that originate from double illuminated confocal volume should fluctuate simultaneously in separate detection channels as the two-color aggregates drift in and out the volume. The cross-correlation between the signals can be obtained e.g. from equation (2). The FFS-based methods can see exclusively the oligomerization of the mobile fraction of the NPM, which is a source of the fluctuation in the signal. To eliminate any crosstalk between channels, we collected the data in the PIE mode that enables entire separation of eGFP and mRFP1 signals. The measuring frame of 84 × 82 pixels was positioned in the nucleoplasm to obtain as homogenous distribution of the fluorescence signal as possible. The cross-correlation brightness B_{cc} was calculated for each pixel in a series of raster images using equation (2). The mean B_{cc} value was then determined across the whole measuring frame. The controls were used for validation of the PIE-ccN&B protocol in live cells. Typical pixel-based distribution of B_{cc} in a cell expressing the R_NPM_G construct is demonstrated in figure 3(A). Typical Gaussian fits obtained for the controls and the G_NPM/R_NPM mix are shown in figure 3(B). Results clearly indicate that the distribution of B_{cc} corresponding to the G_NPM/R_NPM can be found between the distributions corresponding to the positive and the negative controls, being close to the positive one. Mean B_{cc} values calculated for individual cells of the controls and the G_NPM/R_NPM mix ($n = 9$ for the R_NPM_G, $n = 7$ for the G_NPM/R_NPM, $n = 4$ for G_NPM/R) are shown in figure 3(C). The positive control R_NPM_G and the G_NPM/R_NPM mix are significantly separated

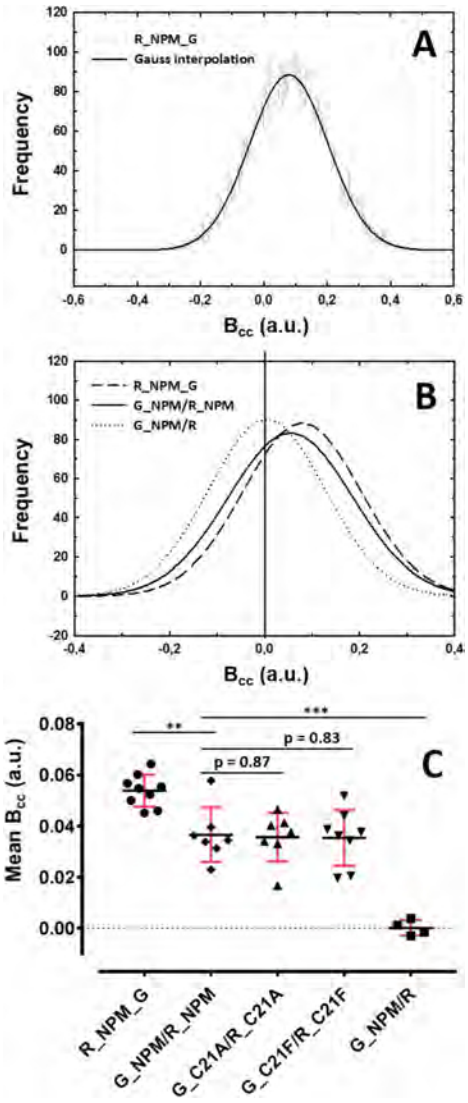

Figure 3. PIE-ccN&B monitoring of the NPM oligomerization; (**A**)—Typical pixel-based B_{cc} distribution of the R_NPM_G construct (positive control) in a single cell. (**B**)—Comparison of typical Gaussian fits of the single-cell distributions acquired for the G_NPM/R_NPM mixture and positive and negative controls. Compared to the negative control G_NPM/R, the G_NPM/R_NPM and the G_NPM_R distributions exhibit significant shift to positive values. (**C**)—Mean B_{cc} values acquired for individual cells expressing the two-color mixtures of WT NPM and the Cys21 mutants are compared with respect to the positive and the negative controls.

from the negative control G_NPM/R. The elevated mean B_{cc} values calculated for the G_NPM/R_NPM mix point to formation of drifting two-color NPM aggregates.

Data acquired for the dual labeled Cys21 NPM mutants in the PIE-ccN&B experiments are presented together with data for the WT NPM in figure 3(C). There is no statistically significant difference between B_{cc} values acquired for the two-color mixtures of NPM mutants and B_{cc} values of the WT protein mixture

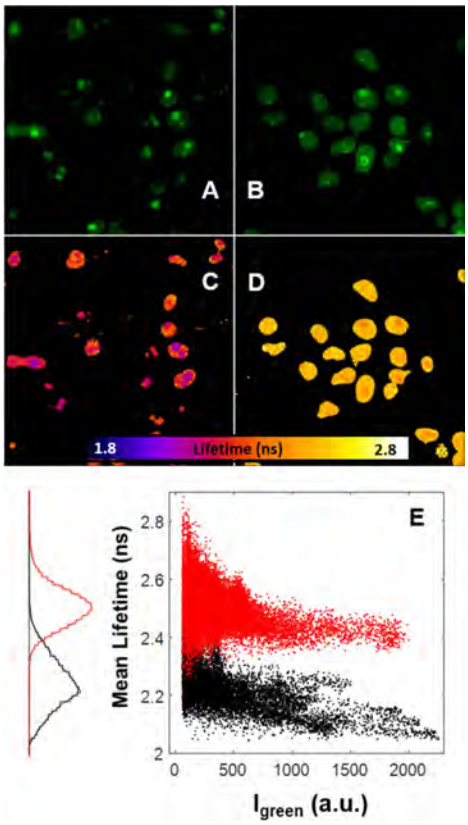

Figure 4. FLIM-FRET monitoring of the NPM oligomerization; Cells expressing G_NPM/R_NPM (**A**) and G_NCL/R_NCL (**B**) were imaged in green channel and their fluorescence was analyzed by the fast-FLIM (**C**), (**D**). The two-color NPM exhibits systematic shortening of the eGFP lifetime (**C**) compared to the two-color NCL (**D**). Pixel-by-pixel correlation analysis of the FLIM images is presented in (**E**). τ_{av} was correlated with fluorescence intensity reflecting eGFP concentration. G_NPM lifetimes (black dots) are significantly below those of G_NCL (red dots). Only pixels with sufficient fluorescence intensities were used for the analysis.

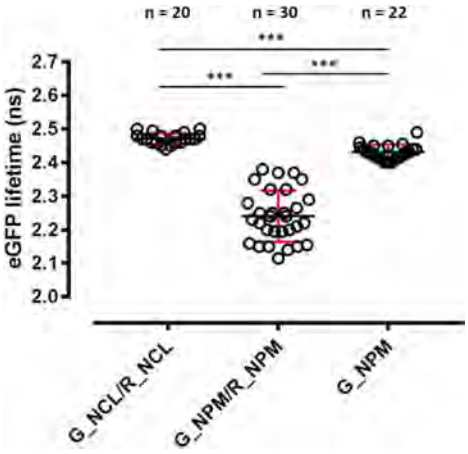

Figure 5. Comparison of eGFP fluorescence lifetimes averaged across the whole nuclei; Individual cells expressing the G_NPM/R_NPM mix are compared to those expressing the G_NCL/R_NCL and G_NPM. FRET is demonstrated by shortening of the eGFP lifetime in cells expressing the G_NPM/R_NPM while the G_NCL/R_NCL reference is close to the G_NPM only.

(p = 0.83 for C21A and 0.87 for C21F). The points obtained for all mixtures are clearly separated from the negative control G_NPM/R. Also this result undermines the inhibitory effect of the Cys21 substitutions on the NPM oligomerization in live cells.

3.3. FLIM-based monitoring of nucleophosmin oligomerization

We applied FLIM-FRET for tracking donor-acceptor proximity in mixed NPM oligomers to confirm independently the oligomerization of the FP-tagged NPM observed in live cells. The proximity should be manifested by shortening of eGFP fluorescence lifetime since excitation is then transferred from the donor eGFP to the acceptor mRFP1. A typical result obtained measuring cells expressing the G_NPM/R_NPM mix is presented in figure 4. NCL like NPM localizes preferentially into the nucleolus [46]. It exhibits also comparable fluorescence intensities in the nuclei of transfected cells. So NCL was used for comparison with NPM in the FLIM-FRET experiments (figures 4(A), (B)). In FLIM images (figures 4(C), (D)), we can clearly see significant shortening of the eGFP lifetime in cells expressing the G_NPM/R_NPM compared to the G_NCL/R_NCL. Pixel-by-pixel correlation analysis of the FLIM images is shown in figure 4(E). Pixel-based average eGFP lifetime τ_{av} is plotted against the corresponding fluorescence intensity. G_NPM/R_NPM τ_{av} values are well below G_NCL/R_NCL values. Therefore the G_NCL/R_NCL mix is used as a negative dual-color reference which helps to demonstrate presence of FRET in the case of the G_NPM/R_NPM.

An alternative analysis of the same data is shown in figure 5. Fluorescence decays were averaged across individual nuclei and rigorously analyzed by least-squares reconvolution analysis yielding nucleus-based mean fluorescence lifetime τ_{mean}. This analysis confirmed significant shortening of the eGFP lifetime in cells expressing the G_NPM/R_NPM mix compared to control cells expressing G_NPM only, i.e. without presence of the acceptor. eGFP lifetimes observed for the G_NCL/R_NCL reference are even slightly longer compared to the G_NPM control. The absence of lifetime shortening comparable to the G_NPM/R_NPM in the G_NCL/R_NCL case indicates that FP crowding in a dense nucleolus environment does not necessarily mean marked eGFP lifetime decrease due to nonspecific FRET.

For further confirmation of the FRET, we performed acceptor photobleaching, which is expected to restore the donor lifetime (figure 6). Two similar dual-color G_NPM/R_NPM cells exhibiting shortened eGFP lifetime were imaged. The mRFP1 acceptor in one of the cells was photobleached by a high-intensity

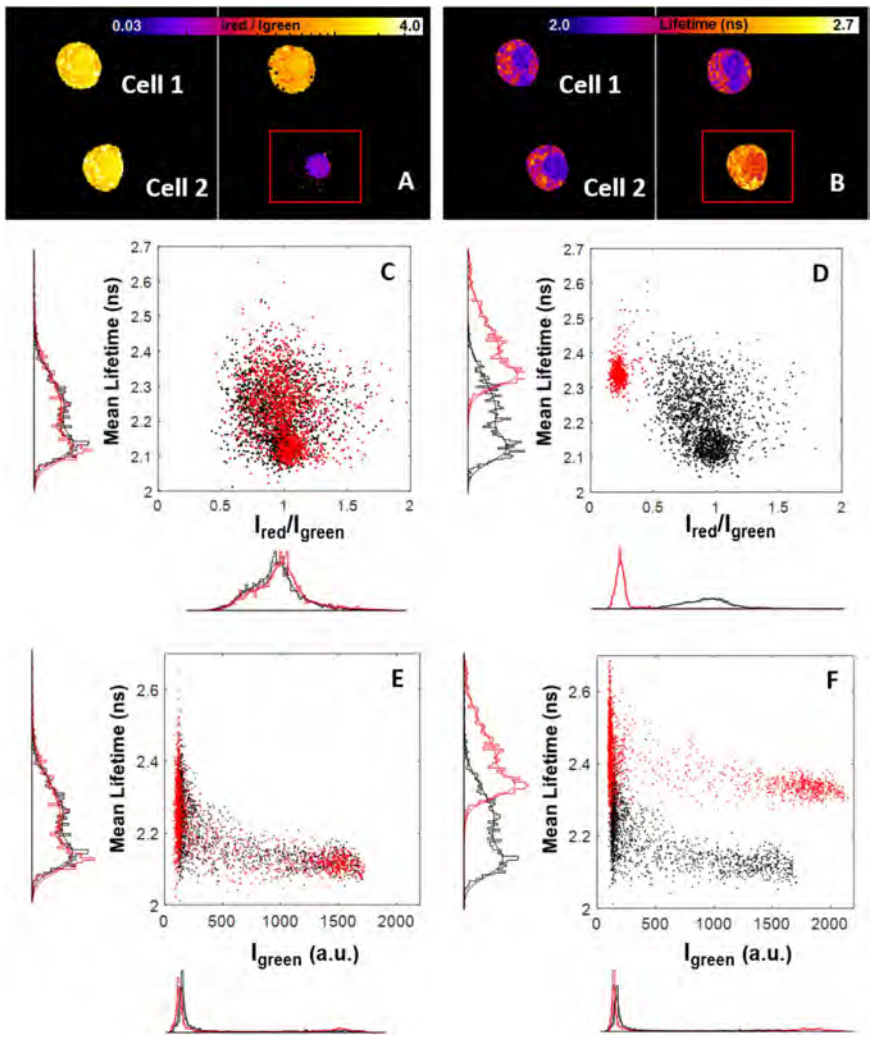

Figure 6. FRET in the G_NPM/R_NPM mix is confirmed by acceptor photobleaching; Two G_NPM/R_NPM cells with similar green and red fluorescence were selected. (**A**)—The red channel intensity in the Cell 2 was reduced during acceptor photobleaching by a strong 550 nm illumination in the area bordered by the red rectangle. (**B**) FLIM images of the cells were measured before and after the photobleaching. Pixel-by-pixel correlation between τ_{av} and red/green intensity ratio before and after photobleaching (**C**, **D**, respectively) and pixel-by-pixel correlation between τ_{av} and eGFP fluorescence intensity before and after photobleaching (**E**, **F**, respectively) was analyzed. Black and red colors correspond to the Cell 1 and the Cell 2, respectively. τ_{av} decreases significantly with increasing I_{red}/I_{green} ratio for both cells prior to photobleaching, which indicates FRET. Then τ_{av} shifts to longer values in the Cell 2 after the photobleaching, which is accompanied by increase in the eGFP fluorescence intensity. This indicates successful FRET disruption.

laser light (550 nm) (figure 6(A)). The eGFP lifetime was restored from ~2.2 ns to ~2.4 ns as seen in the FLIM images (figure 6(B)), which corresponds to the lifetimes presented in figure 5. The pixel-by-pixel analysis of eGFP lifetimes in dependence on acceptor/donor intensity ratio (figures 6(C), (D)) and in dependence on eGFP intensity (figures 6(E), (F)) clearly shows that donor lifetimes and intensities shifted after the acceptor bleaching, which indicates FRET confirming the donor/acceptor proximity. Therefore in accordance with the PIE-ccN&B data, the observed FRET points to proximity of the labeled NPM in hetero-aggregates formed in live cells. We attribute the observed aggregates to the NPM oligomerization expected in live cells.

3.4. Effect of Cys21-mutations on the NPM oligomerization

Having validated the *in vivo* FLIM-FRET method with the WT NPM, we used it to investigate the influence of the Cys21 substitutions on the NPM oligomerization in live cells. Pixel-by-pixel correlation analysis of FLIM images showed that eGFP lifetimes have similar distribution in cells expressing the WT and the Cys21-mutated NPM (figures 7(A)–(C), respectively). The significant lifetime decrease with increasing donor/acceptor ratio points to FRET in mixed oligomers

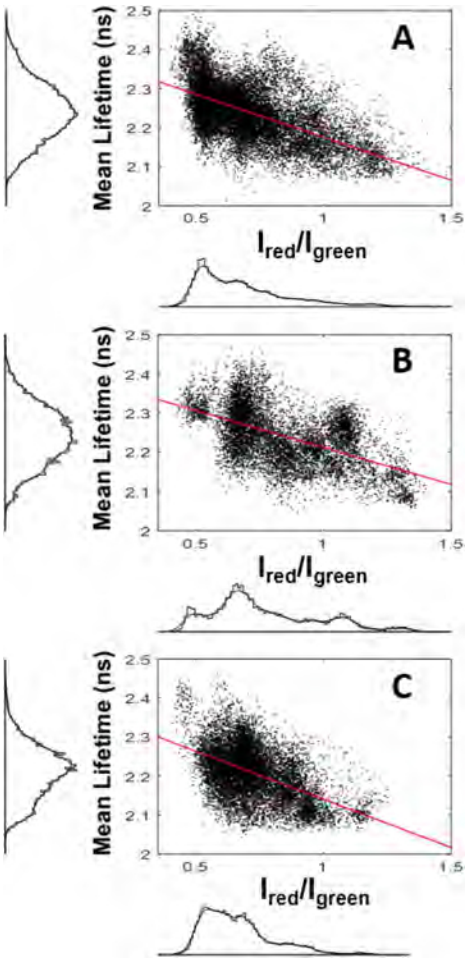

Figure 7. Oligomerization of the Cys21-NPM mutants in live cells observed by the FLIM-FRET; Lifetime correlation plots and corresponding histograms for cells expressing the two-color mixtures of WT NPM (**A**), NPM-C21A (**B**) and NPM-C21F (**C**) are shown. FP-tagged proteins were localized in cells as shown in figure 1. Intensity-thresholded FLIM images were analyzed pixel-by-pixel to correlate τ_{av} with the ratio of fluorescence signals registered in the red and the green channel, the I_{red}/I_{green} corresponds to acceptor/donor ratio. Red lines are linear fits visualizing slope in the plots.

regardless from what FP-tagged NPM variant the oligomers are formed.

Both the FLIM-FRET and the PIE-ccN&B experiments reveal that the Cys21-mutants exhibit oligomerization behavior *in vivo* similar to the WT NPM. To further support these findings, we applied *in vitro* co-immunoprecipitation utilizing a pull-down of eGFP-labeled proteins by the GFP-Trap beads [27]. The co-immunoprecipitation can be used for identification of interaction partners of the eGFP-labeled NPM. It should therefore detect endogenous NPM co-residing in the hetero-oligomers.

We transfected cells with plasmids for expression of free eGFP, G_NPM, G_NPM-C21A and G_NPM-C21F. The eGFP-labeled proteins are co-immunoprecipitated with their interaction partners (figure 8). The endogenous NPM is detected in each precipitate which points to tight interaction of the native NPM with the G_NPM and with both of the eGFP-tagged Cys21-NPM mutants. No interaction is found in the control sample containing free eGFP. The GFP-Trap proves that the eGFP-labeled NPM is able to form complexes with the endogenous NPM irrespective of the Cys21 mutations, which were expected originally to inhibit the NPM oligomerization.

4. Discussion

With an aim to study a role of the NPM oligomerization in cell response to cytotoxic drugs and in launching the apoptosis, we apply gentle noninvasive dual-color confocal microscopy techniques, ccN&B and FLIM-FRET, for detection of NPM oligomers. The ccN&B was combined with the PIE to suppress the spectral cross-talk caused by bleeding of the eGFP fluorescence to the mRFP1 detection. However, use of this combined approach meant longer time spent on one pixel (100 μs/pixel) during scanning, which is needed for acquiring a sufficient number of photons to calculate a cross-correlation parameter (B_{cc}) with lower experimental dispersion. Even after prolonging the dwell time, the number of photons was not high enough to perform brightness-based analysis in both colors, e.g. B_{cc} plots in dependence on values of one-color brightness corresponding to the green and the red channels [42]. Additionally as mentioned in the Introduction, it is impossible to guarantee single-molecule conditions with standard fluorescent proteins and transfection protocols in live cells, which impedes the proper FFS-based analysis. Moreover, marked photobleaching together with FRET further disqualify the quantitative ccN&B analysis in the herein presented system. Therefore, by using the reliable positive and negative controls, we show qualitatively that the PIE-ccN&B detects reliably joint diffusion of green and red tags residing in mixed aggregates (figure 3).

The PIE-ccN&B experiments are complemented with the FLIM-FRET method to confirm the detection of the FP-tagged NPM mixed aggregates. Shortening of the eGFP lifetime is observed in cells expressing the G_NPM/R_NPM mix. Appearance of FRET is verified by measuring reference not-exhibiting FRET (G_NCL/R_NCL) with very similar cellular expression and localization (figures 4, 5) and by acceptor photobleaching (figure 6). FRET indicates proximity of eGFP and mRFP tags in the mixed aggregates. Therefore, the B_{cc} elevation indicating joint diffusion observed in the PIE-ccN&B and the eGFP lifetime shortening due to FRET monitored by the FLIM are attributed to formation of oligomers in the G_NPM/R_NPM-expressing cells. The PIE-ccN&B experiments monitored the oligomerization in nucleoplasm where NPM is in a dynamic equilibrium with the

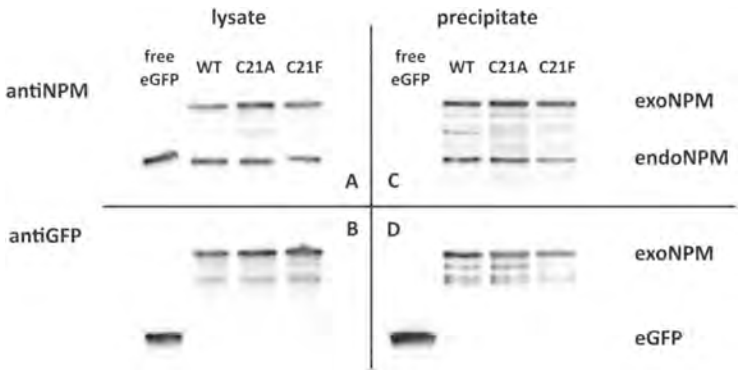

Figure 8. GFP-Trap assay; HEK-293T cells were transfected with plasmids for expression of G_NPM, G_NPM-C21A and G_NPM-C21F. Cells expressing free eGFP served as a control. Cell lysates were tested for NPM by anti-NPM antibody (**A**) and for GFP by anti-GFP (**B**). GFP-pulled immunoprecipitates were tested for the endogenous NPM (**C**). Free eGFP in control lane and GFP-tagged NPM in other lanes were detected by the anti-GFP antibody (**D**). Endogenous NPM co-precipitates with all the eGFP-tagged NPM variants indicating formation of hetero-complexes. No NPM is detected in the control lane.

nucleolus [30, 59]. In FLIM, the NPM oligomerization was detected both in the nucleoplasm and in the nucleolus.

Having validated the *in vivo* fluorescence-based methods, we planned to investigate response of these methods to the oligomerization inhibition. Among artificial drugs, NSC348884 was reported to inhibit the NPM oligomer formation [33]. However, massive apoptosis was induced by the NSC348884 concentrations inhibiting the oligomer formation [33], which impeded its use for reduction of the oligomerization. The affected cells detached from the bottom cover glass (our observation) making it inappropriate reference for the dual-color FFS monitoring of oligomerization in the adherent live cells. Moreover, identical drug dosage induces the apoptosis also in peripheral blood lymphocytes of healthy donors (our unpublished results) making it inappropriate for use in cancer treatment. Therefore, we investigated the influence of Cys21 mutations that were also reported to inhibit the NPM oligomerization *in vitro* [16, 25].

A crucial role of the Cys21 located in the N-terminus of NPM for oligomerization has been suggested by *in vitro* SDS-PAGE electrophoresis substituting the Cys21 with Ala or Phe [16, 25]. In parallel, preferential localization of WT NPM to the nucleolus was shown to be affected by its oligomerization status. The correlation between the NPM oligomerization and its localization to the nucleolus was postulated using Myc-tagged mutants and immunofluorescence staining [31]. Hence, compromising the oligomerization with the substitutions in Cys21 should lead to delocalization of NPM to the nucleoplasm. Surprisingly, we have not observed the expected delocalization of the NPM-C21A and NPM-C21F FP-tagged mutants. The localization of these mutants was indistinguishable from the localization of the tagged WT NPM (figure 1). This suggests that either the inhibition does not occur or the inhibition does not result in delocalization of NPM to the nucleoplasm.

In agreement with the localization microscopy, the herein presented advanced confocal microscopy methods, the PIE-ccN&B (figure 3) and the FLIM-FRET (figure 7), confirm that the NPM-C21A and the NPM-C21F mutants can reside in joint oligomers under physiological conditions in live cells. All these experiments thus indicate that the Cys21 mutations do not inhibit the NPM oligomerization in live cells. In our laboratory, we came to similar conclusion also in experiments addressing the delocalization of the Cys21 NPM mutants to the cytoplasm driven by interaction with the FP-tagged NPMc+ (data not shown). The NPMc+ induced partial cytoplasmic delocalization of WT NPM [27] but also of the Cys21 mutants. The delocalization indicates that all the investigated variants of NPM interact with the NPMc+ probably in mixed aggregates that facilitate joint drifting inside the cell.

For verification whether the eGFP-tagged Cys21 mutants as well as the WT NPM interact with the endogenous unlabeled protein, we applied *in vitro* co-immunoprecipitation using the pull down of eGFP-labeled proteins by the GFP-Trap beads [27]. The immunoprecipitation confirms the interaction (figure 8). This result together with the results obtained by the *in vivo* methods explain why the nucleolar localization of the NPM Cys21 mutants was not compromised in our experiments.

In our opinion, the *in vitro* SDS-PAGE electrophoresis used for detection of the oligomers in [16, 25] provides information on oligomer instability under rather harsh conditions of the SDS-PAGE. It would not be surprising if the presence of the detergent affects preferentially the stability of the oligomers formed from the mutated NPM. Thus, the electrophoresis can yield biased results on the NPM oligomers formation since the observed instability might

have no obvious consequence under the physiological conditions inside live cells.

5. Conclusions

We adopted dual-color methods based on modern fluorescence confocal microscopy, the PIE-ccN&B and the FLIM-FRET, for monitoring of the NPM oligomerization in live cells expressing dual-color NPM. We observed that Cys21 substitutions do not necessarily inhibit the oligomerization under physiological conditions. The results obtained by the *in vivo* approaches were independently supported *in vitro* by the GFP-Trap immunoprecipitation assay. Application of the FLIM- and the FFS-based methods for monitoring of the NPM oligomerization is our original innovation and should have deeper consequences in understanding of AML-related cell biochemistry. The *in vivo* monitoring of potential targets for the anticancer therapy [28, 29] is of high importance and should offer a way to study the effect of targeted anticancer drugs directly in live cells. The approaches presented here should be of general utility in studying oligomerization and interactions of nuclear and nucleolar proteins.

Acknowledgments

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8.3 Příloha č. 3: AML-associated mutation of nucleophosmin compromises its interaction with nucleolin

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AML-associated mutation of nucleophosmin compromises its interaction with nucleolin

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ABSTRACT

C-terminal mutations of the nucleolar protein nucleophosmin (NPM) are the most frequent genetic aberration detected in acute myeloid leukemia (AML) with normal karyotype. The mutations cause aberrant cytoplasmic localization of NPM and lead to loss of functions associated with NPM nucleolar localization, e.g. in ribosome biogenesis or DNA-damage repair. NPM has many interaction partners and some of them were proved to interact also with the mutated form (NPMmut) and due to this interaction thereby to be withdrawn from their site of action. We analyzed the impact of the mutation on NPM interaction with nucleolin (NCL) which is also prevalently localized into the nucleolus and cooperates with wild-type NPM (NPMwt) in many cellular processes. We revealed that the NCL-NPM complex formation is completely abolished by the mutation and that the presence/absence of the interaction is not affected by drugs causing genotoxic stress or differentiation. Deregulation resulting from changes of NCL/NPMwt ratio may contribute to leukemogenesis.

1. Introduction

Nucleolin (NCL, C23) and nucleophosmin (NPM, B23) are abundant nucleolar phosphoproteins whose roles in ribosomal biogenesis, cell cycle, DNA-damage repair or apoptosis were widely reported (Leary and Huang, 2001; Scott and Oeffinger, 2016; Yang et al., 2002). NPM is localized mainly in the nucleolus due to nucleolar localization signal (NoLS) at its C-terminus (Falini et al., 2007) as well as to multiple interactions of its N-terminus with arginine-rich motifs in ribosomal proteins, which are produced in the granular component (GC) of the nucleolus (Mitrea et al., 2016). NCL probably uses a bipartite nuclear localization signal (NLS) to enter the nucleus and then accumulates within the nucleolus by virtue of binding to other nucleolar components (Schmidt-Zachmann and Nigg, 1993). NPM is often denoted as one of the agents mediating the NCL nucleolar localization (Korgaonkar et al., 2005).

Nucleophosmin dynamically shuttles between nucleoli and the nucleoplasm serving as a chaperone for nucleic acids, histones and non-histone proteins in many cellular processes. NPM occurs mainly in oligomers located in the nucleoli, whereas a form phosphorylated on Thr199 is present in the cytoplasm during mitosis and prevents aberrant centrosome duplication (Tokuyama et al., 2001). Recently, Mitrea et al (Mitrea et al., 2014) reported that the phosphorylation of particular sites in NPM N-terminal domain caused dissociation of folded pentamers into disordered monomers. Aberrant cytoplasmic localization of NPM was detected in acute myeloid leukemia (AML) patients with specific C-terminal mutation of NPM (NPMmut, in about one-third of all AML cases, rare in MDS and not detected in other cancer types or healthy individuals) affecting the NoLS (Falini et al., 2005). The most frequent mutations (type A, B or D) are characterized by loss of two crucial tryptophans, W288 and W290, and almost complete loss of nucleolar localization. Several minor mutations, i.e. type E, lack only W290 and their nucleolar localization is partially retained (Brodska et al., 2017). The results of our previous study indicated that the presence of NPM mutations may be associated with an efficient immune response against NPM or against its interaction partners (Kuzelova et al., 2015).

Nucleolin is the most abundant nucleolar protein, it represents about 10% of the nucleolar protein content (Durut and Sáez-Vásquez, 2015). Above ninety percent of cellular NCL is located in the nucleolus (Scherl et al., 2002) and it was found to function in nucleolus formation (Ma et al., 2007). However, it also operates in the nucleoplasm, in the cytoplasm and on the cell surface (Scott and Oeffinger, 2016). Cell-surface NCL was detected prevalently in cancer cells and is supposed to act in tumorigenesis (Gattoni-Celli et al., 2009). Therefore, immunoagents targeting the NCL are currently widely tested in research and clinical trials (Gilles et al., 2016; Koutsioumpa and Papadimitriou., 2014; Palmieri et al., 2015; Rosenberg et al., 2014).

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The NPM and NCL function as chaperons and they share a large number of their interaction partners (Scott and Oeffinger, 2016). As they operate mainly in the nucleolus, they interact with many ribosomal proteins (Mitrea et al., 2016). However, proteins involved in apoptosis (i.e. p14Arf, p53, mdm2) (Nalabothula et al., 2010; Takagi et al., 2005), DNA-damage repair (i.e. APE1, HAUSP) (Lim et al., 2015; Vascotto et al., 2009) or centrosome duplication also frequently interact with them. Recently, a small ligand of cell-surface NCL, N6L (Destouches et al., 2012), was proved to inhibit NPM oligomerization through direct interaction with its N-terminal domain (De Cola et al., 2018). Both NCL and NPM co-immunoprecipitated with aprataxin regardless of pre-treatment with DNAse or RNAse, suggesting that nucleic acid is not required for the complex formation (Becherel et al., 2006).

Generally, it is supposed that the oligomerization domain of NPM serves also as interaction interface for binding other proteins (De Cola et al., 2018). Colombo et al. (Colombo et al., 2006) reported that p14Arf, a tumor suppressor which was shown to interact with NPM through the N-terminal domain (Enomoto et al., 2006), interacts also with NPMmut. Moreover, this interaction was found to cause Arf delocalization to the cytoplasm according to aberrant localization of NPMmut. However, the C-terminal (187-241aa) region of recombinant NPM was identified as the binding site for NCL in GST-pull down assays (Li et al., 1996). In NCL, aa540-628 were detected as the region responsible for the interaction with NPM. The complex was preserved in genotoxic stress conditions represented by actinomycin D (actD) treatment in HeLa cells, but it was reported to disappear in cells synchronized to mitotic phase (Liu and Yung, 1999). However, the NCL-NPM association in a complex with another nucleolar protein, fibrillarin, was later detected by immunoprecipitation in synchronized mitotic HeLa cells (Ma et al., 2007). The relocalization of NPM interaction partners due to its leukemia-associated mutation may affect the function as well as the accessibility of interacting proteins for therapeutic intervention. The impact of C-terminal NPM alterations on NPM-NCL interaction is therefore of high importance and to our knowledge it has not been tested yet.

All-trans retinoic acid (ATRA) was reported to cause NPM nuclear relocalization and degradation of NPMmut (El Hajj et al., 2015; Martelli et al., 2015). Concurrently, ATRA-induced nuclear redistribution of cytoplasmic NCL was observed in leukemia cell line HL-60 (Otake et al., 2005). With regard to its relevance for the clinical practice, it is important to analyze the fate of the NPM-NCL complex after ATRA treatment

We confirmed the interaction between NPM and NCL in intact and actD-treated cells and we showed, that NCL does not interact with AML-related NPM mutant A. To our knowledge, the nucleolin is the first protein whose interaction with NPM was proved to be compromised by the NPM mutation. None of actinomycin D, Leptomycin B or ATRA treatment affected the NPM-NCL interaction, but we observed increased NPM oligomer formation induced by ATRA. We also found that NCL expression is not changed in cells with the NPM mutation.

2. Material and methods

2.1. Cell culture and chemicals

Cancer cell line HEK-293 T (gift from dr. Němečková, Institute of Hematology and Blood Transfusion, the line was authenticated by short tandem repeats analysis in May 2016) was cultivated in DMEM (Sigma-Aldrich) with 10% FBS (Biochrom, Germany), leukemic cell lines OCI-AML2 and OCI-AML3 (both DSMZ, Germany) were cultivated in alpha-MEM (Sigma-Aldrich) with 20% FBS. Peripheral blood mononuclear cells from leukapheretic products of AML patients were separated using Histopaque 1077, washed with PBS, resuspended in RPMI1640 (Sigma-Aldrich). All cells were cultivated in 5% CO_2 atmosphere at 37 °C. All patients signed informed consent to the use of their biological material for research purposes in agreement with the Declaration of Helsinki. The Ethics Committee of the Institute of Hematology and Blood Transfusion approved this research at the application of grant No 16-30268 A. All samples were tested for the presence of C-terminal NPM mutation by PCR and the mutation type was determined by sequencing (Kuzelova et al., 2015). Stock solutions of 10μM actinomycin D (actD), 5 μM Leptomycin B (LmB) and 100 μM ATRA (all from Sigma-Aldrich) were added to cell suspensions to final concentrations 5 nM (actD), 10 nM (LmB) and 1 μM (ATRA) respectively for times indicated in the Results section.

2.2. Transfection

As described in detail previously (Brodska et al., 2016, 2017), gene for NPM and NCL were amplified from cDNA library (Jurkat cells, Origene) by PCR and inserted to vectors peGFP-C2 and pmRFP1-C2 (originally Clontech) designed for expression of protein chimeras with a fluorescent protein connected to the N-terminus of the target protein by standard methods of molecular cloning. NPM mutants were constructed by PCR using extended primers containing mutated part of exon 12 of the *NPM1* gene and restriction sites (Brodska et al., 2017). After amplification in E. coli, the plasmids with subcloned genes were purified with PureYield Plasmid Miniprep System (Promega) and transfected into adherent cell lines using jetPRIME transfection reagent (Polyplus Transfection).

2.3. Cell lysis and western blotting

2.3.1. Cell lysis

Cells were washed with PBS and lysed depending on the intended application. For direct use in SDS-PAGE, the cells were lysed in Laemmli sample buffer (SB, 50 mM Tris pH 6.8, 2% SDS, 100 mM DTT, 10% glycerol), boiled at 95 °C for 5 min, centrifuged at 200.000 *g*/4 °C for 4 h and the supernatant was stored at −20 °C. For other applications, the cells were lysed in Lysis buffer (LB, 10 mM Tris/Cl pH7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, protease and phosphatase inhibitors) for 30 min/4 °C, centrifuged at 20.000 *g*/4 °C for 10 min and supernatant was mixed 1:1 with appropriate buffer.

Native and semi-native PAGE: Lysates were mixed with 2xnative buffer (NB, 50 mM Tris pH6.8, 10 mM DTT, 10% glycerol) and without boiling subjected to 7,5% AA Tris-glycin gel without SDS for native ELFO or to the gel with SDS (2%) for semi-native ELFO.

SDS-PAGE: Lysates were mixed with 2xSB, boiled for 5 min and stored at −20 °C until used for 125% acrylamide (AA) SDS-PAGE.

2.3.2. Western blotting

Five to ten microliters of each sample were subjected to native or SDS-PAGE and transferred into PVDF membrane (BioRad). Mouse monoclonal antibodies against β-actin, GFP, dsRed, NCL and NPM (clone 3F291 for NPMwt + mut detection, clone E3 for NPMwt detection) were from Santa Cruz Biotechnology. All mouse primary antibodies were used at a dilution 1:100-1:500. Rabbit antibody against NPMmut (pab50321, Covalab) was used at 1:2000 dilution. Anti-mouse and anti-rabbit HRP-conjugated secondary antibodies were purchased from Thermo Scientific and used at concentrations 1:10.000-1:50.000. ECL Plus Western Blotting Detection System (GE Healthcare) was used for chemiluminescence visualization and evaluation by G-box iChemi XT4 digital imaging device (Syngene Europe). Alternatively, Alexa488-conjugated anti-rabbit and Alexa647-conjugated anti-mouse secondary antibodies (ThermoFisher) for simultaneous detection of NPMmut and wt + mut were used.

2.4. Immunoprecipitation

Immunoprecipitation using GFP- or RFP-Trap (Chromotek) was performed according to manufacturer´s instruction as described previously (Brodska et al., 2017). Briefly, cells were harvested and washed

with PBS, lysed in LB for 30 min/4 °C and centrifuged at 20.000 *g*/4 °C for 10 min. Lysate was mixed with GFP/RFP-nanobeads and rotated for 1 h/4 °C. Then the beads were extensively washed with diluting buffer (10 mM Tris/Cl pH7.5, 150 mM NaCl, 0.5 mM EDTA), resuspended in SB, boiled at 95 °C for 10 min and centrifuged 20.000 *g*/4 °C for 10 min. Supernatant was stored at −20 °C until used for SDS-PAGE.

2.5. Confocal microscopy

2.5.1. Live-cell imaging

Subcellular distribution and colocalization of eGFP- or mRFP1-fused variants of NPM and NCL was observed by Olympus FluoView FV1000 confocal microscope (Olympus Corporation). For subcellular distribution statistics, at least 500 cells from three independent experiments were evaluated. Fluorescence images were processed by FluoView software FV10-ASW 3.1.

2.5.2. Immunofluorescence

The samples were prepared as described previously (Brodska et al., 2016). Briefly, cells in suspension were seeded on a coverslip in humidified chamber for 15 min and then fixed with 4% paraformaldehyde (PFA) overnight at 4 °C. After 10 min of permeabilization by 0,5% Triton X-100, the cells were incubated for 1 h with a rabbit anti-NPMmut primary antibody (Covalab, 1:200) and for another 1 h with the secondary antibody (Alexa-Fluor488-conjugated anti-rabbit, Life Technologies, 1:200) and with Hoechst33342 (1 μM, Life Technologies). For nucleoli staining, primary AlexaFluor647-conjugated anti-NPM (ab202578, Abcam, 1:200) directed to detect central part of NPM and visualising prevalently wild-type NPM form was used. The stained cells were observed under confocal laser scanning microscope FluoView FV1000 (Olympus Corporation).

2.6. Statistical analyses

As described in our previous work (Brodska et al., 2017), the majority of experiments were performed using cell lines and repeated until the observed differences between groups reached statistical significance. Primary samples from AML patients available at the Institute of Hematology and Blood Transfusion were analyzed as well. A p-value of 0.05 or lower was pre-set to be indicative of a statistically significant difference between groups compared. In diagrams, arithmetic means of at least three replicates of all experiments were plotted with SD error bars. Significance levels (p values of ANOVA or Student´s *t*-test) were determined using InStat Software (GraphPad Software).

3. Results

3.1. Stability of NPM oligomers

NPM occurs *in vivo* mainly in pentamers and we have previously reported that heterooligomers containing both NPM wild type (NPMwt) and NPMmut mutually affect the localization of individual NPM variants (Brodska et al., 2017). The oligomers can be detected by native electrophoresis (Fig. 1a) and they are partially preserved even in SDS-PAGE conditions when native lysis conditions are used (semi-native PAGE, see section 2.3.1.). Under these semi-native conditions, bands from oligomers as well as from monomers were visible in immunoblot (Fig. 1b). The ratio of monomeric/oligomeric form detected by semi-native PAGE was significantly enhanced for NPMmut variant suggesting lowered stability of NPMmut-formed oligomers as compared to the NPMwt ones (Fig. 1c).

3.2. Interaction with NCL

Heterooligomer formation, e.g. the interaction between NPMwt and NPMmut, was formerly proved by GFP-immunoprecipitation (GFP-Trap) from HEK-293 T cells transfected with GFP-fused NPM variants (Brodska et al., 2017). Interestingly, endogenous NCL, a known NPM-interacting partner, was detected in precipitates of NPMwt, but not in these of NPMmut (Fig. 2a). Therefore, we co-transfected HEK-293 T cells with GFP-labeled NCL and RFP-labeled NPMwt/NPMmutA and immunoprecipitated GFP-NCL with its interaction partners. Indeed, while endogenous NPMwt precipitated in all samples, the RFP-NPMwt, but not the RFP-NPMmutA was detected by RFP or NPM antibodies (Fig. 2b) indicating the loss of NCL-NPM interaction caused by C-terminal NPM mutation.

In Fig. 2a, a very weak band in anti-NCL blot was detected in the sample transfected with NPMmutE. This indicates the possibility of partially maintained NCL interaction with NPM mutant type E. We thus analyzed localization of fluorescently labeled NCL and NPM by confocal microscopy (Fig. 3a). As expected, localization of NPMwt was clearly

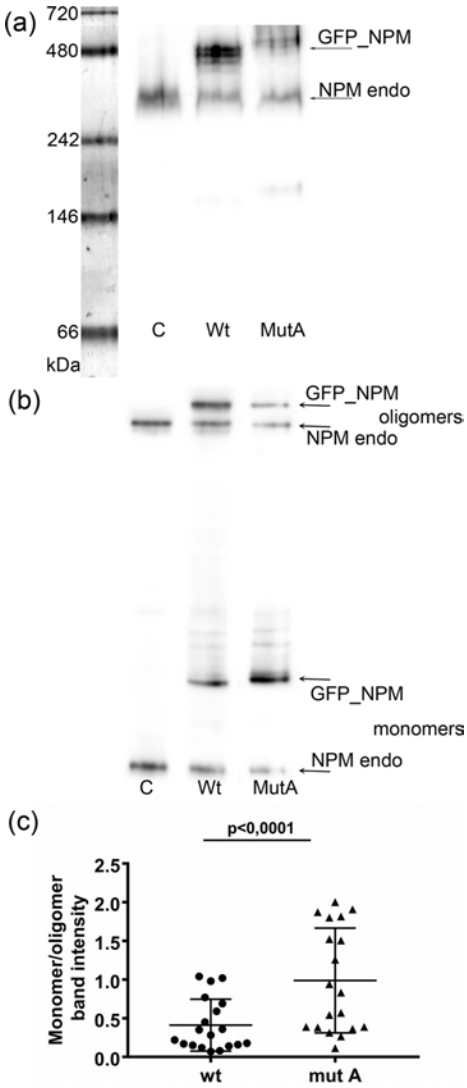

Fig. 1. Native (a) and semi-native (b) PAGE of NPM detection in lysates of HEK-293 T cells transfected with GFP_NPMwt (lane Wt) or GFP_NPMmutA (lane MutA). lane C: control (untransfected) cells. (c) Statistical evaluation of GFP_NPM monomer vs. oligomer band intensities from semi-native PAGE blots. The ratio for NPMwt (circles) and NPMmut (triangles) is significantly different as evaluated by paired *t*-test.

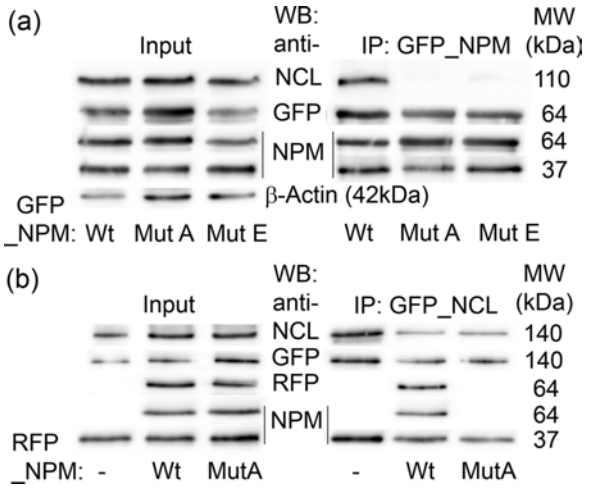

Fig. 2. GFP-Trap immunoprecipitation of HEK-293 T cells transfected with GFP_NPM (64 kDa) variants (a) or co-transfected with GFP_NCL (140 kDa) and RFP_NPM (64 kDa) variants (b). Fusion proteins were detected by anti-GFP/RFP as well as by anti-NPM/NCL antibodies.

affected by the presence of mutated NPM variants (mutA or mutE). On the other hand, no presence of NCL in the cytoplasm was detected (Fig. 3b). Conversely, NPMmutA was abundantly present in the nucleoli of cells co-transfected with NPMwt but not with NCL. Localization characteristics of NPMmutE are shifted to partial nucleolar occurence as compared to mutA (Brodska et al., 2017), nonetheless the fraction of cells with NPMmutE in the nucleoli was further increased in NPMwt co-transfected cells (Fig. 3b). Co-immunoprecipitation experiments focused on both labeled proteins in the co-transfected cells also confirmed absence of the NCL-NPMmutA interaction (Fig. 3c). Controversial results were obtained from samples cotransfected with NCL and NPMmutE. While no GFP_NPMmutE was detected in RFP precipitates, a band from RFP_NCL was sometimes detected in the GFP precipitates from the same sample. In these experiments, the presence of endogenous NPM (37 kDa) in precipitates served as control.

3.3. Cytotoxic drugs treatment

The NCL was proved to interact with NPM through the 187-241aa region (Li et al., 1996), which is retained in leukemia-related NPM mutants. The absence of NPMmutA interaction with NCL may be thus caused either by conformational alterations of the mutated NPM or by physical separation of the proteins due to NPMmut aberrant localization. The latter possibility could rationalize also the observed retention of NCL-NPMmutE interaction. Several drugs were used to induce partial colocalization of NCL and NPMmut and their interaction in treated cells was examined. Both NCL and NPM were shown to relocalize into the nucleoplasm after actD treatment (20 nM actD for 8 h), although the localization pattern of individual proteins stayed partially different in HeLa cells (Brodska et al., 2016). Similar images were obtained also for HEK-293 T cells treated with low-dose (5 nM) actD overnight (Fig. 4a). Another way to bring NPMmut close to NCL is to use an inhibitor of the nuclear exporter Crm1, e.g. Leptomycin B (LmB). LmB was reported to cause NPMmut redistribution from the cytoplasm to the nucleus (Falini et al., 2006). Addition of 10 nM LmB caused complete NPMmut relocation during 40 min (Fig. 4a). Immunoprecipitation proved persisting NCL-NPMwt interaction and no NCL-NPMmut interaction in both actD and LmB-treated cells (Fig. 4b) demonstrating that relocalization of NPMmut close to NCL did not re-establish the interaction. Hence, the absence of the interaction is likely not caused by distinct localization of the partners due to NPM mutation.

3.4. ATRA-induced changes in NPM expression and stability

As all-trans retinoic acid (ATRA) treatment was also reported to affect NPM localization and NPMmut stability (Martelli et al., 2015), we tested the effect of ATRA addition on NPM variants localization, expression and interaction with NCL. Partial redistribution of NPMmut into the nucleoli was observed after 48 h exposition of transfected HEK-293 T cells to 1μM ATRA (Fig. 4c). Accordingly to results with actD and LmB, NCL-NPMwt, but not NCL-NPMmut interaction was detected by immunoprecipitation. However, contrary to recent studies (El Hajj et al., 2015; Martelli et al., 2015), we did not observe NPMmut degradation caused by ATRA in the experiments with fluorescently labeled NPM forms transfected into HEK-293 T cell line. Therefore, we tested leukemia cell lines OCI-AML2 (NPMwt) and OCI-AML3 (NPMmutA) as well as samples from AML patients for localization and expression of NPM. The nucleoli differed in their shape and number between samples with and without NPM mutation. Less numerous and less round-shaped nucleoli detected in cells with NPMmutA changed their characteristics and were closer to those of NPMwt phenotype in response to ATRA treatment (Fig. S1). In NPMmut-expressing samples, the localization of NPMmut was partly shifted from the cytoplasm to the nucleus after 48 h of 1μM ATRA treatment (Fig. 5a). Only moderate attenuation of both total NPM and NPMmut expression was caused by ATRA (Fig. 5b). To closely approximate the conditions used by Martelli et al (Martelli et al., 2015), we transferred OCI-AML3 from standard alpha-MEM with 20%FBS into 10%FBS. We also tested various lysis protocols (see Section 2.3.1.), as we experienced that standard lysis procedure caused partial degradation of NPM with shorter product detected at about 20 kDa (Fig. S2). None of these changes led to detection of an extensive NPMmut degradation in samples exposed to ATRA. Moreover, ATRA treatment showed significant attenuation of 20 kDa band, in particular in blots visualised by anti-NPMmut antibody. To further investigate this phenomenon, we subjected control and ATRA-treated samples to semi-native PAGE (Figs. 5c and S3). Stabilization of NPM oligomers by ATRA was detected in both OCI-AML2 and OCI-AML3 cell lines regardless the FBS percentage (see enhanced bands at higher MW and attenuated signal at lower MW in ATRA-treated samples). In OCI-AML3 this effect was detected also by anti-NPMmut antibody irrespective of FBS content (Fig. S2). Accordingly with other studies reporting on ATRA-induced differentiation (Brown and Hughes, 2018) we detected significantly increased fraction of cells in G0/G1 phase in ATRA-treated cells (Fig. 5d).

3.5. NCL expression

As the NCL and NPM were reported to cooperate in ribosome biogenesis, cell cycle progression or nucleic acid binding during DNA damage repair (Amin et al., 2008; Leary and Huang, 2001; Ma et al., 2007; Scott and Oeffinger, 2016; Yang et al., 2002), changes in their relative concentrations may deregulate these processes. We evaluated expression of NPM and NCL in cells in dependence on their NPM mutational status (illustrative western-blots are shown in Fig. 6a). The amount of NCL did not depend significantly on NPM status (Fig. 6b). On the other hand, cells expressing NPMmut had lower NPM total protein level (Fig. 6c). As the mutated form of NPM does not interact with NCL, the amount of NPM available for complex formation with NCL is further reduced in these cells. The impact of enhanced NCL/NPMwt ratio in nucleoli of NPMmut-positive cells on tumorigenesis warrants further studies.

4. Discussion

We investigated the effect of leukemia-related NPM mutation on its oligomeric status and on the interaction with its main nucleolar partner, NCL. Depletion of NPM in cancer cells was reported to lead to loss of nucleolar compactness and to deformation of its round shape.

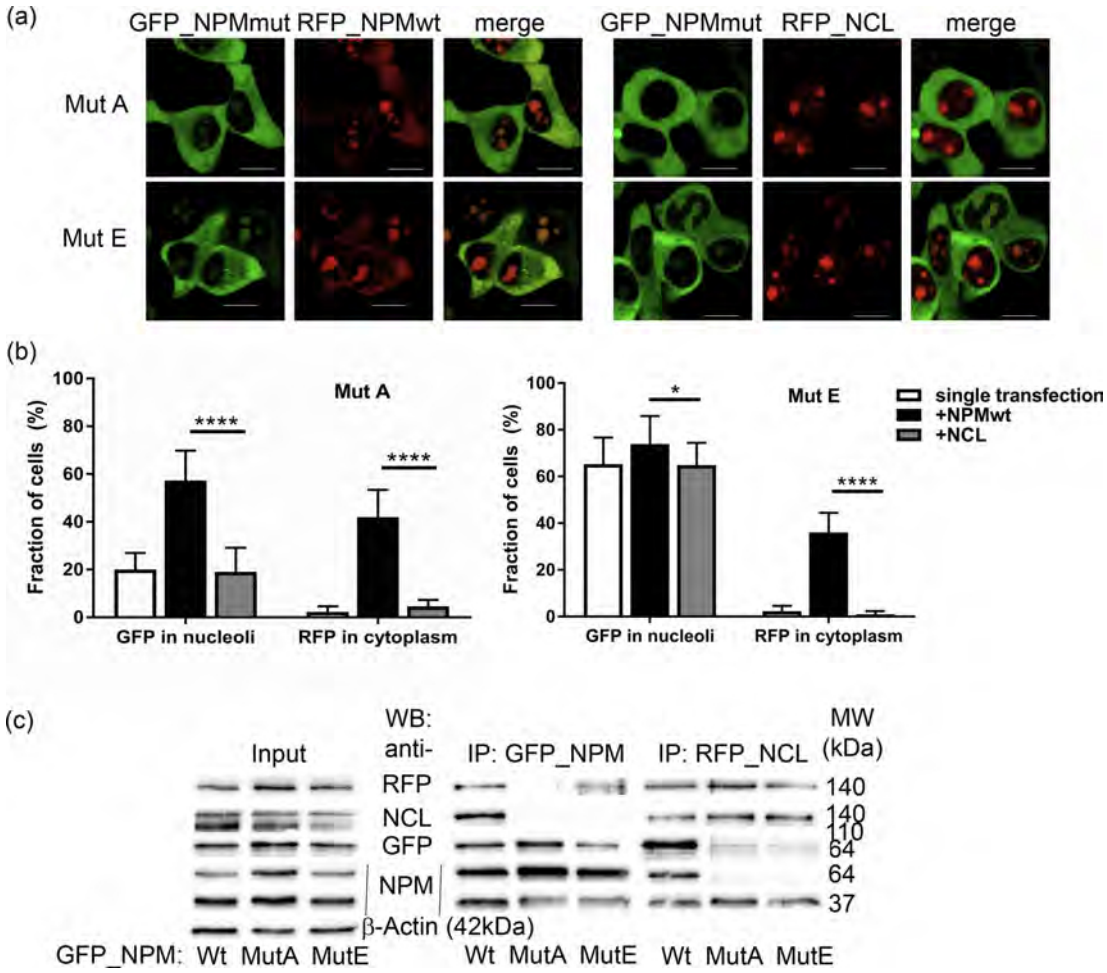

Fig. 3. Analysis of HEK-293Tcells co-transfected with GFP_NPMmut and RFP_NPMwt or RFP_NCL. (a) Localization of GFP_NPMmut variants (green) and RFP_NPMwt or RFP_NCL (red) imaged by confocal microscope. Bar represents 10μm. (b) Statistical analysis of the presence of GFP signal (NPMmut) in the nucleoli and of RFP signal (NPMwt or NCL) in the cytoplasm of transfected cells. White bars: control single-transfected cells (NPMmut only for GFP evaluation, NPMwt or NCL only for RFP analysis), black bars: cells co-transfected with GFP_NPMmut and RFP_NPMwt, grey bars: cells co-transfected with GFP_NPMmut and RFP_NCL. Student´s *t*-test revealed significant difference (*p < 0,05; ****p < 0,0001) between NPMwt and NCL as to their ability to affect the intracellular distribution of NPMmut. (c) Detection of NCL-NPM interaction in GFP- and RFP-immunoprecipitates of transfected proteins. Fusion proteins were detected by anti-GFP/RFP as well as by anti-NPM/NCL antibodies.

Besides the nucleolar distortion, rearrangement of perinucleolar heterochromatin was observed in NPM-depleted cells (Holmberg Olausson et al., 2014). Therefore, destabilization of nucleolar stucture can be expected also in cells with reduced amount of NPM in the nucleolus due to the mutation. By native electrophoresis experiments we demonstrated that the ratio between oligomeric and monomeric NPM form was significantly attenuated for NPMmut (Fig. 1). Therefore, although the NPMmut keeps the ability to form oligomers, stability of complexes formed by NPMmut is lower than the stability of complexes with NPMwt and interaction of NPM with other proteins may be thus also affected by the mutation.

The NCL-NPM interaction was demonstrated in intact and actD-treated HeLa cells (Liu and Yung, 1999). In their paper, the authors observed loss of the interaction in mitotic cells, although cell-free kinase assay revealed that the loss of interaction did not result from specific NPM phosphorylation during mitosis. The results from western blots showed low NCL expression in cells synchronized to mitotic phase and our microcopy observation confirmed low fluorescence intensity of RFP-tagged NCL from mitotic cells (unpublished results). Therefore we presume that the absence of NPM band in NCL precipitate from mitotic cells could be due to insufficient amount of precipitated NCL. Indeed, several years later, the NCL-NPM interaction was proved also for mitotic HeLa cells (Ma et al., 2007). In our present experiments, we confirmed the interaction of NCL with NPMwt. On the other hand, NPM type E mutation substantially reduced NCL-NPM complex formation and we did not detect any interaction between NCL and NPM when type A mutation was present (Figs. 2 and 3). The aminoacid sequence involved in NPMwt interaction with NCL (aa187-241) is not directly affected by the AML-related mutation. We therefore tested the hypothesis that aberrant localization of NPMmut prohibits its interaction with NCL. Using cytotoxic drugs affecting the localization of NPM (actD, LmB) we demonstrated that NCL-NPMwt interaction was preserved under genotoxic stress conditions (Fig. 4). Nonetheless, relocalization of NPMmutA to close proximity with NCL did not re-establish the interaction between these proteins. Further study of the reasons for the interference of AML-related NPM mutation with NCL-NPM interaction, including e.g. possible conformational changes, is needed as it could be associated with a loss of function of NCL-NPM complexes and

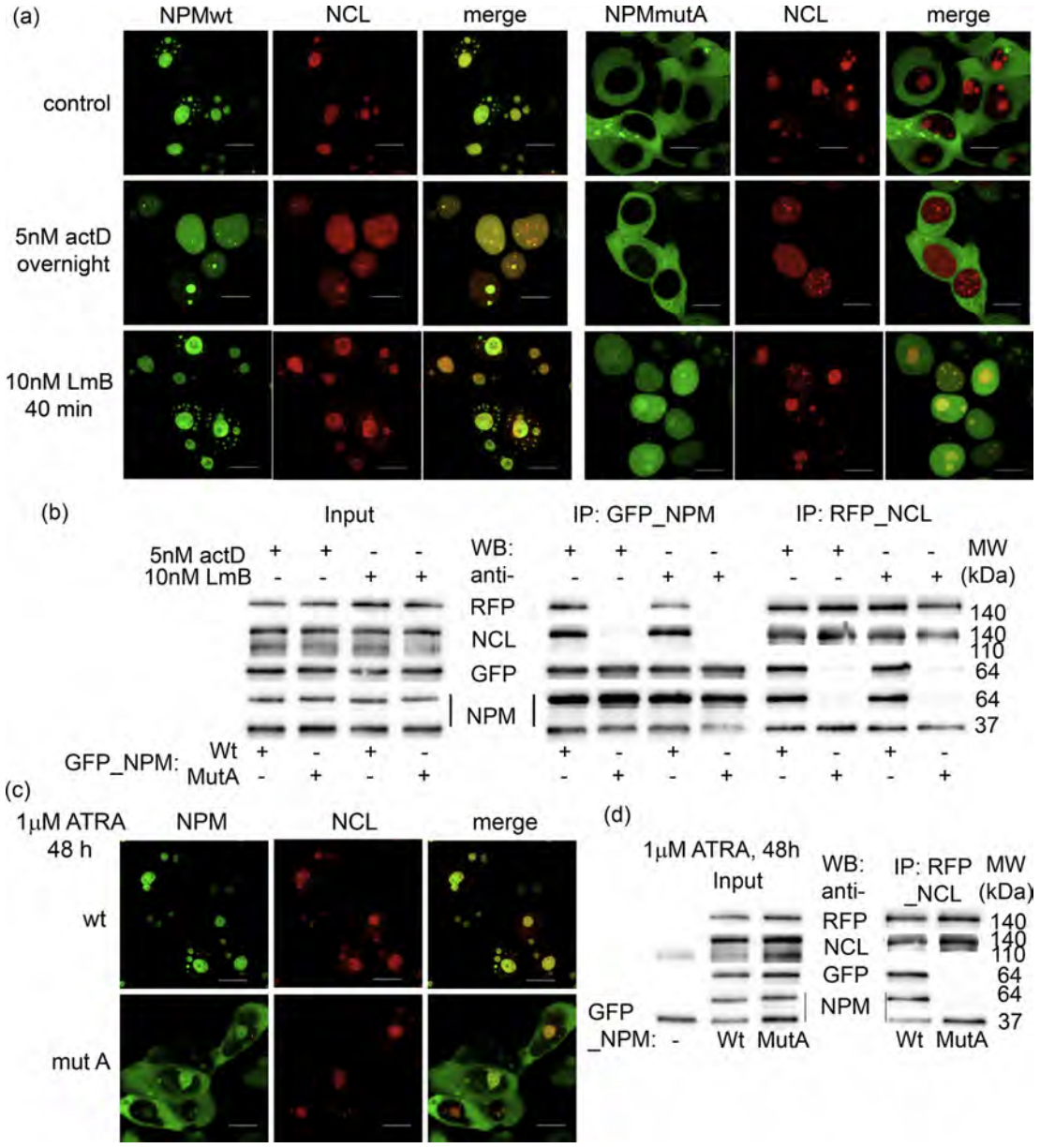

Fig. 4. Analysis of cytotoxic drugs effect on NCL-NPM interaction in HEK-293 T cells co-transfected with GFP_NPMmutA or GFP_NPMwt and RFP_NCL. (a) Localization of GFP_NPMmutA or GFP_NPMwt (green) and RFP_NCL (red) in intact and actD or LmB-treated cells. Bar represents 10μm. (b) Detection of NCL-NPM interaction in GFP- and RFP-immunoprecipitates from actD or LmB-treated cells. (c) Effect of ATRA treatment on GFP_NPMmutA or GFP_NPMwt (green) and RFP_NCL (red) localization. Bar represents 10μm. (d) Detection of NCL-NPM interaction in RFP-immunoprecipitates of transfected cells.

contribute to leukemogenesis.

Several NPM interaction partners (e.g. p14Arf, APE1 or many ribosomal proteins) were documented to interact with NPM through its oligomerization domain and some of them were reported to interact also with NPMmutA. Delocalization of these proteins from their site of action due to interaction with aberrantly localized NPMmut likely compromises their pathways and activity. One of the most important interaction partners of NPM is the tumor suppressor p53 and its ability to activate its downstream targets p21, Puma and WIG1 was reported to be attenuated in NPM depleted cells (Holmberg Olausson et al., 2014). Lower expression of NPMwt due to the presence of NPMmut may thus partly inactivate induction of p53 signalization and contribute to genomic instability. On the other hand, we demonstrated that NCL did not interact with NPMmutA and its expression was not changed in cells containing NPMmut (Fig. 6). The NCL expression has been found unchanged also in NPM-depleted cells where the NCL immunostaining was used to confirm the more irregular shape of nucleoli (Holmberg Olausson et al., 2014) which was found also by us in NPMmut-containing cells. NCL and NPM cooperate in many cellular processes and overexpression or knockout of one of them affects the activity of the other one. High level of NCL in reference to NPM nucleolar expression in cells with NPMmut may thus lead to unbalance of these two proteins in processes affected by their mutual expression (i.e. ribosome biogenesis, genotoxic stress response etc). NCL overexpression was found

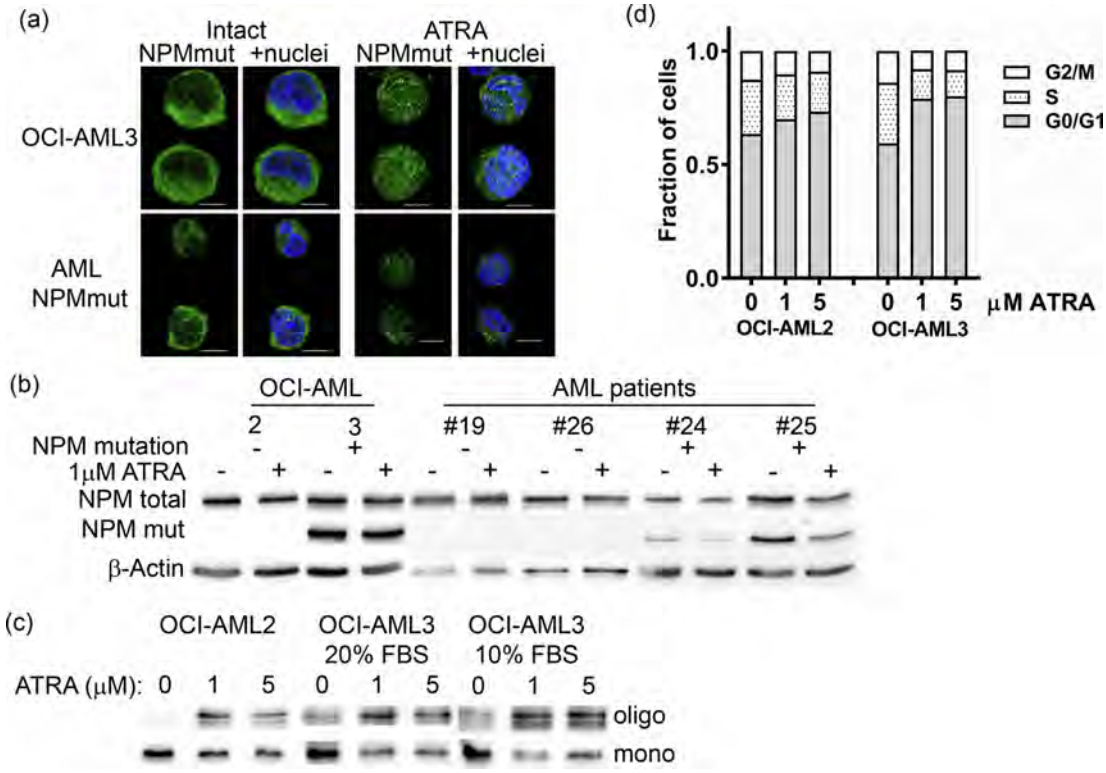

Fig. 5. NPM expression in ATRA-treated leukemic cell lines and cells from AML-patients. (a) Localization of NPMmut (Alexa488, green) in intact or 1µM ATRA-treated cells. Nuclei are visualised by Hoechst33342 (blue). Bar represents 5µm. (b) Expression of total NPM or NPMmut in control or ATRA-treated cells. Expression of β-Actin served as a load control. (c) NPM oligomer/monomer expression at semi-native PAGE of lysates from control cells and from cells treated by 1 or 5 µM ATRA. (d) cell cycle analysis of ATRA effect on OCI-AML cell lines. In all experiments, ATRA was added for 48 h.

in multiple tumor types (Otake et al., 2007; Qiu et al., 2013; Wolfson et al., 2018) and overexpression of NCL was proved to correlate with worse prognosis (Chen et al., 2016; Fonseca et al., 2015). Therefore, the study of impact of altered nucleolar NCL/NPM ratio in cells with NPMmut on cell proliferation and apoptosis is appealing.

ATRA is successfuly used to treat acute promyelocytic leukemia (APL) and several clinical studies of its treating potential in other AML types are in progress. In particular, epigenetic modifiers were reported to sensitize leukemia patients to ATRA treatment (van Gils et al., 2017). It was previously reported that 1µM ATRA causes degradation of NPMmut and re-entry of delocalized NPMwt from cytoplasm to the nucleus (Martelli et al., 2015). We detected partial relocalization of NPMmut from the cytoplasm to the nucleoli/nuclei in all samples, i. e. in transfected HEK-293 T cells, in leukemic cell lines and in blasts of AML patients treated in vitro with ATRA (Figs. 4 and 5). We confirmed also in this case the difference between NPMwt and NPMmut in the ability to interact with NCL. However, we did not detect any substantial degradation of NPMmut (Figs. 5 and S2). A moderate decrease of not only NPMmut but total NPM expression was observed in certain samples after ATRA treatment. Concurrently, cell cycle analysis showed an enhanced fraction of cells in G0/G1 phase, which is probably associated with the known differentiating effect of ATRA. Slightly attenuated NPM expression may thus be accounted to dampened cell proliferation and suppressed ribosomal synthesis. Interestingly, together with these changes, we detected increased NPM oligomer fraction in ATRA-treated cells. This effect was visible also by specific NPMmut antibody documenting that stabilization but not degradation of NPMmut is induced by ATRA treatment (Fig. S3). Lower fraction of mitotic cells (or cells in G2/M phase) in ATRA-treated samples may contribute to enhanced oligomer/monomer ratio due to higher proportion of phosphorylated NPM monomers in mitotic phase (Mitrea et al., 2014). This hypothesis may be supported by structural analysis of a similar protein from nucleoplasmin/nucleophosmin family, nucleoplasmin (NP), which infers that the stability of pentamers created by the active (phosphorylated) form decreased although the crystal structure of the phosphorylated NP core domain could be superimposed onto the wild-type NP (Taneva et al., 2008). We also attempted to inhibit NPM oligomerization using NSC348884 (Balusu et al., 2011), but we were not able to reproduce experiments described by Balusu et al. due to massive apoptosis triggered by NSC348884 prior to any change in oligomerization (data not shown). Interestingly, in our recent work (Holoubek et al., 2018) we detected an interaction between NPM oligomerization mutant C21 F, where Cysteine21 was replaced with Phenylalanine (Prinos et al., 2011), and NPMwt *in vivo* by advanced fluorescence techniques suggesting there is a difference in NPM oligomerization status, or at least at its detection limits, within the living cells and within their lysates.

5. Conclusion

Nucleolin and nucleophosmin belong to the main players in many essential cellular processes including ribosome biogenesis, genotoxic stress response, proliferation and apoptosis. C-terminal mutation of NPM is the most frequent aberration in acute myeloid leukemia and although widely studied, its contribution to leukemogenesis remains unclear. We documented that NPM mutation led to loss of NPM interaction with NCL and that the absence of the NCL-NPMmut interaction was not caused by different localization of NPMmut. We further showed that NCL expression is not affected by the presence of NPM mutation suggesting that NCL/NPM ratio in nucleoli is substantially increased in cells with NPMmut. This difference can lead to aberrant cell

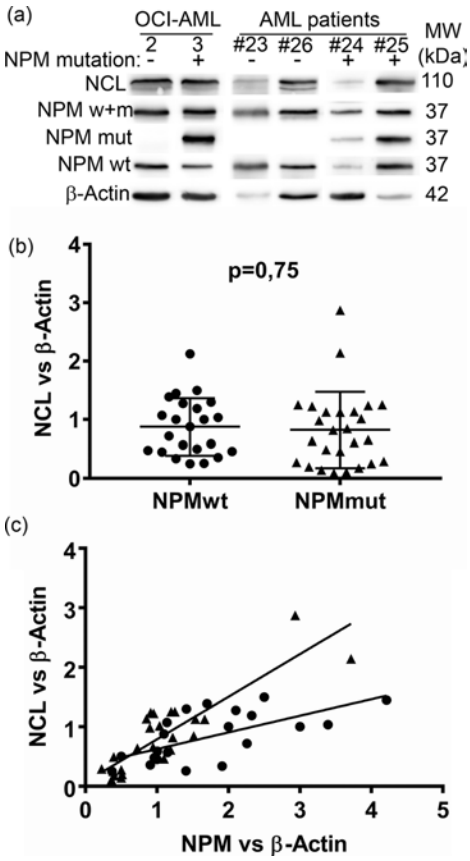

Fig. 6. Expression of NCL and NPM in samples from cell lines and AML-patients with NPMwt (circles) and NPMmut (triangles). (a) immunoblot illustration of NCL and NPM variants expression. (b) statistical analysis of NCL expression in cells with different NPM status and (c) NCL and NPM expressions relative to β-actin expression.

signalization and may thus contribute to leukemogenesis. On the other hand, we proved that NCL-NPMwt interaction persisted under genotoxic stress as well as in differentiating conditions after ATRA treatment. Moreover, stabilization of NPM oligomers was observed in ATRA-treated leukemic cells.

Acknowledgement

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Declarations of interest: none.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2018.08.008.

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Dodatkové informace k publikaci č. 3: AML-associated mutation of nucleophosmin compromises its interaction with nucleolin

Supplementary material

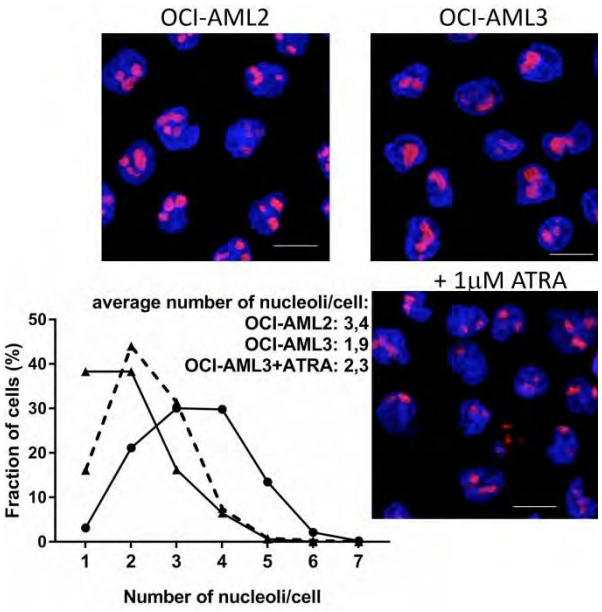

Fig. S1: NPM expression in leukemic cell lines OCI-AML2 (NPMwt) and OCI-AML3 (NPMmutA). Localization of NPM in intact or 1μM ATRA-treated cells was detected by Alexa647-conjugated primary anti-NPM (red). Nuclei are stained with Hoechst33342 (blue). Bar represents 10μm. Graph: Distribution of number of nucleoli/cell in leukemic cell lines with (OCI-AML3, triangles) or without (OCI-AML2, circles) NPMmut. Solid line: intact cells, dashed line: OCI-AML3 treated by 1μM ATRA for 48h. For this graph, at least 300 cells in each sample was analyzed.

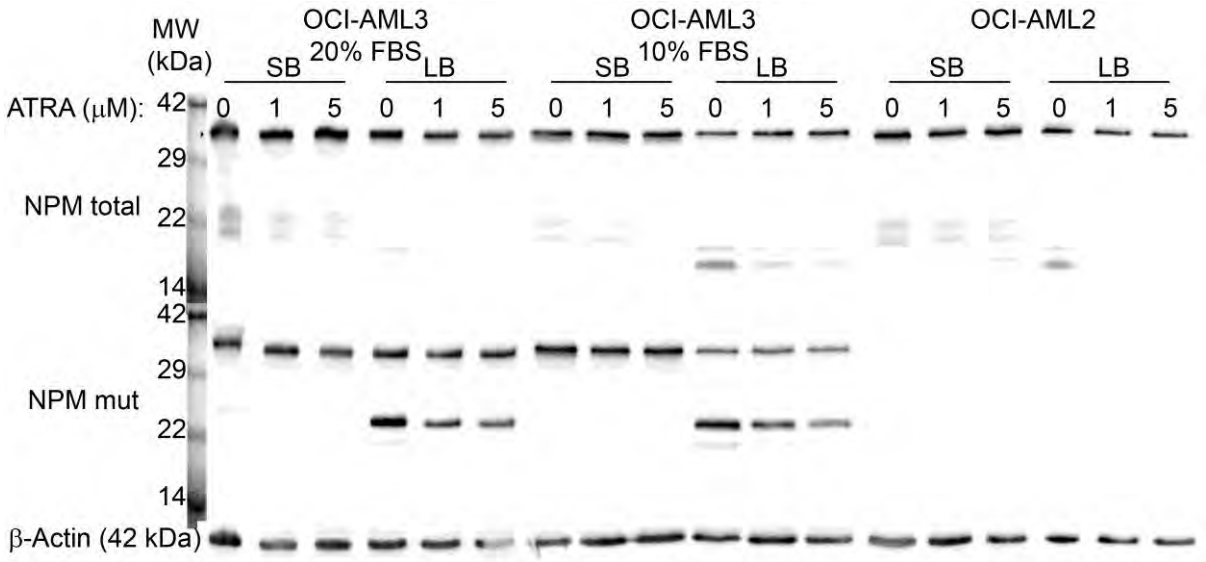

Fig. S2: Effect of lysis procedure and cultivation conditions on immunoblot NPM detection. Expression of total NPM or NPMmut in control or ATRA-treated cells was investigated in lysates from cells cultivated in 10 or 20% FBS. Lysis directly to sample buffer (SB) or to lytic buffer following by dilution to SB (LB) were compared. Expression of β-Actin served as a load control. ATRA in concentrations as indicated was added for 48h.

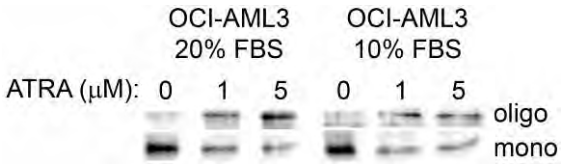

Fig. S3: NPMmut oligomer/monomer expression at semi-native PAGE of native OCI-AML3 lysates from control cells and from cells treated by 1 or 5 μM ATRA cultivated in media with various FBS percentage.

8.4 Příloha č. 4: NSC348884 cytotoxicity is not mediated by inhibition of nucleophosmin oligomerization

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NSC348884 cytotoxicity is not mediated by inhibition of nucleophosmin oligomerization

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Nucleophosmin (NPM) mutations causing its export from the nucleoli to the cytoplasm are frequent in acute myeloid leukemia (AML). Due to heterooligomerization of wild type NPM with the AML-related mutant, the wild-type becomes misplaced from the nucleoli and its functions are significantly altered. Dissociation of NPM heterooligomers may thus restore the proper localization and function of wild-type NPM. NSC348884 is supposed to act as a potent inhibitor of NPM oligomerization. The effect of NSC348884 on the NPM oligomerization was thoroughly examined by fluorescence lifetime imaging with utilization of FRET and by a set of immunoprecipitation and electrophoretic methods. Leukemia-derived cell lines and primary AML cells as well as cells transfected with fluorescently labeled NPM forms were investigated. Our results clearly demonstrate that NSC348884 does not inhibit formation of NPM oligomers neither in vivo nor in vitro. Instead, we document that NSC348884 cytotoxicity is rather associated with modified cell adhesion signaling. The cytotoxic mechanism of NSC348884 has therefore to be reconsidered.

Abbreviations

NPMwt	Wild-type nucleophosmin
NPMmut	Nucleophosmin with AML-associated mutation type A
C21	Cysteine 21 in nucleophosmin
C21A	Substitution of C21 to alanine
C21F	Substitution of C21 to phenylalanine
Δ25	Nucleophosmin with deletion of the first 25 amino acids
Δ100	Nucleophosmin with deletion of the first 100 amino acids
Δ117	Nucleophosmin with deletion of the first 117 amino acids
G_	EGFP-labeled protein at the N-terminus
R_	MRFP1-labeled protein at the N-terminus

The human *NPM1* gene is located on the chromosome 5q35 and encodes a 32.6 kDa polypeptide. Nucleophosmin (NPM), encoded by the *NPM1* gene, is a ubiquitously expressed phosphoprotein residing predominantly in the granular component of the nucleolus and shuttling dynamically among nucleoli, the nucleoplasm and the cytoplasm[1–4]. It functions as a chaperone[5] and is engaged in regulation of various cellular processes including the ribosome biogenesis[6], DNA-damage repair[7], centrosome duplication[8], and DNA replication[9]. Furthermore, NPM is involved in apoptosis and can modulate p53 stability and activity[1,10].

NPM overexpression, fusion, or mutation have oncogenic potential and are associated with cancer progression in many types of solid tumors[11] and in hematopoietic malignancies[12–17]. Acute myeloid leukemia (AML) with mutated *NPM1* accounts for about 1/3 of de novo adult AML, and *NPM1* is the most frequently mutated gene in AML with normal karyotype (50–60% incidence)[12]. To date, more than 100 *NPM1* mutation types have been identified in AML, occurring almost exclusively in the last exon (exon 12) of the gene[18]. A nucleotide insertion and/or deletion (indel mutations) lead to the frame shift in the region encoding the C-terminus of NPM. All these *NPM1* mutations result in loss of tryptophan residues at positions 288 and/or 290, which form the main part of the nucleolar localization signal (NoLS) ensuring nucleolar localization of the wild-type NPM (NPMwt). Moreover, the indel mutations frequently generate an additional nuclear export sequence (NES), which labels the mutated protein for the nuclear exporter XPO1 and targets NPM into the cytoplasm[19]. Both the loss of NoLS and

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the occurrence of the new NES lead to the accumulation of the NPM mutant (NPMmut) in the cytoplasm[12,20,21]. Cytoplasmic NPM serves as an immunohistochemical marker with prognostic relevance[22–25], and is also associated with reduced incidence of some HLA class I alleles, possibly due to anti-leukemia immune response[26,27]. In the absence of an additional genetic aberration, AML patients with *NPM1* mutation have better response to intensive chemotherapy.

NPM forms pentamers, which may assemble into decamers, through a conserved N-terminal domain[28–31]. This domain plays a crucial role in NPM interactions with many of its partners, e.g. with p14Arf or c-myc[1,32,33]. NPM with C-terminal mutation has been reported to retain the ability to form oligomers[34,35]. Therefore heterooligomers consisting of both NPMmut and NPMwt are frequent and the localization of individual variants is mutually affecting each other[20,36]. This results in a decrease of NPMwt concentration in the nucleolus and may thus cause a loss of function of the fraction of NPMwt delocalized into the cytoplasm[37]. Interaction of NPMmut with tumor suppressors also leads to aberrant transfer of these proteins into the cytoplasm and, presumably, to the loss or restriction of their biological function[38,39]. However, the role of the NPM mutation and of protein delocalization in AML initiation and the treatment response has not been elucidated yet.

In general, two approaches are tested to prevent delocalization of NPMwt and of its interaction partners together with the NPMmut into the cytoplasm. First, an inhibition of NPM nuclear exporter XPO1, and, the second, an impairment of the NPM oligomerization. Both approaches aim to reestablish the correct NPMwt localization[40,41]. A known XPO1 inhibitor Leptomycin B can block NPM transport into the cytoplasm, but it cannot be used for AML treatment owing to a high toxicity[25]. Alternative second-generation XPO1 inhibitors, selinexor and eltanexor, are drugs with promising anticancer effect. Selinexor is currently being tested in a phase I clinical trial[42]. Recently, its effect on complex formation between NPMmut and the transcription factor PU.1 with a key role in monocyte lineage differentiation has been demonstrated[43].

In the present study, we focus on the second option, i.e. on the manipulation of NPM localization by an interference with NPM oligomerization. NPM oligomerization is mediated by its N-terminal domain. The equilibrium between the pentameric and monomeric forms is ruled mainly by posttranslational modifications, namely phosphorylation of numerous phosphosites[44], and by interactions with proteins affecting NPM folding and assembly, in particular with p14Arf[45]. Recent findings suggest a role of the N-terminal region in nucleolar NPM localization linked to its interaction with proteins containing arginine-rich linear motifs and with ribosomal RNA[46]. This complements the original concept that NPM molecules are directed to the nucleolus by the nucleolar localization signal (NoLS) located in the very C-terminus of NPM[35]. In NPM mutants associated with AML, the alterations of the C-terminus result in loss of the NoLS. Consequently, AML-associated NPM mutations cause changes in the tertiary structure of the C-terminus[47] that is responsible for significant aggregation tendency[48]. Although the C-terminal mutation was previously documented not to abrogate the NPM oligomerization ability[35], we revealed that oligomers formed by NPMmut tend to dissociate into monomers more likely than oligomers formed by NPMwt[49]. Therefore, therapy based on the interference with NPM oligomerization might be beneficial for AML patients with *NPM1* mutation, as only NPMmut-consisting oligomers would be inhibited under optimal conditions and NPMwt could retain its function.

The small molecule NSC348884 was reported to prevent formation of NPM oligomers[50]. However, the action of NSC348884 was found to be rather complex. NSC348884 activates p53, inhibits cell growth, and triggers apoptosis[41,50]. In a number of recent works the effects of NSC348884 treatment are being ascribed in particular to "NPM inhibition"[51–53] and authors by default assume that NSC348884 inhibits NPM oligomerization, as declared[41,50]. Nonetheless, in a set of cancer cell lines our experiments targeting NPM oligomerization by NSC348884 systematically exhibited surprisingly low effectiveness of the drug in this respect. Therefore we decided to focus on this single aspect of NSC348884 and to rigorously investigate its potential to inhibit NPM oligomerization both in vivo and in vitro.

To study the effect of NSC348884, it is crucial to validate a set of reliable methods for detection of NPM oligomers in cell lysates and living cells as well as to find trustable controls. The FLIM-FRET, native electrophoresis and immunoprecipitation, were therefore methods of choice for complex evaluation of the NSC348884 action. A set of NPM-mutants served as a control for a coherent validation of these methods and for assessment of their sensitivity to follow NPM oligomerization.

Having established a robust experimental and control system, we analyzed the effect of NSC348884 on NPM oligomerization in cell lysates and living cells. The effect of NSC348884 on proliferation and apoptosis is presented as well. In addition, unexpected significant NSC348884-induced decrease of cell adhesivity is described and a putative mechanism of NSC348884 action is proposed.

Results

Interaction and stability of NPM with C21 point mutation. Evaluation of NSC348884 ability to affect NPM oligomerization in living cells and cell lysates requires sensitive methods for oligomerization monitoring as well as reliable positive and negative controls. To closely simulate the native conditions, we preferentially searched for point mutations that were reported to inhibit NPM oligomerization.

Point mutation in C21 was shown to be important for NPM oligomerization[54]. Recently, we have shown that conclusions about oligomerization of these mutants obtained under rather harsh conditions of the SDS-PAGE[54] might strongly differ from results obtained in living cells by fluorescence lifetime imaging (FLIM-FRET) and by immunoprecipitation in lysates[55]. We demonstrated that some C21 NPM-mutants, originally expected not to oligomerize[34,54], are actually able to form complexes with the endogenous NPM in living cells. In addition, immunoprecipitation revealed interaction of the endogenous NPM with eGFP-labeled NPM bearing point mutation at C21 (G_C21), i.e. where C21 was substituted either to Ala (G_C21A), or to Phe (G_C21F). In the present work, we used fluorescence microscopy and native electrophoresis to characterize in detail the impact of

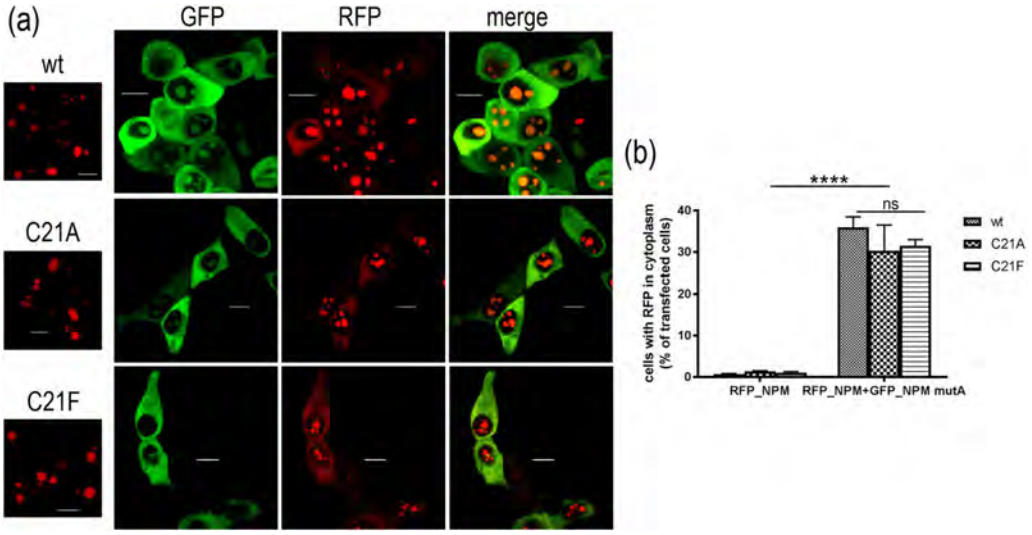

Figure 1. Interaction between NPMmut and C21 point mutants. (**a**) Left: localization of R_NPMwt/C21A/C21F in single transfected 293T cells. Right: 293T cells co-transfected with G_NPMmut (green) and R_NPMwt/C21A/C21F variants (red). Red signal in the cytoplasm and green signal in nucleoli witness for an interaction between NPMmut and NPMwt/C21A/C21F. (**b**) Statistical evaluation of the subcellular localization of NPMwt and C21 mutants. Fraction of transfected 293T cells displaying red signal from the cytoplasm in single transfected cells (left group) and in cells co-transfected with G_NPMmut (right group). Error bars represent ± SD of at least 3 independent experiments, $p < 0.0001$ (****) for the differences between the co-transfected and the single-transfected cells.

these C21 mutations. Both G_C21 mutants exhibited nucleolar localization, identical to that of NPMwt (Fig. 1). Furthermore, the mRFP1-labeled NPM_C21 (R_C21) was found in the cytoplasm of HEK-293T (293T) cells co-transfected with eGFP-labeled NPMmut (G_NPMmut). The fraction of cells exhibiting mRFP1 signal in the cytoplasm was comparable for all R_NPM variants (Fig. 1). Both C21 mutants therefore seem to form heterooligomers with NPMmut, alike NPMwt in living cells.

To independently verify these findings, we examined native lysates from cells expressing G_C21A or G_C21F by semi-native and native electrophoresis[49]. Briefly, the lysates from cells harvested into non-reducing, non-denaturing buffer were directly subjected without boiling to acrylamide gel with and without SDS, respectively (see Material and Methods). These relatively gentle separation methods allowed for detection of both NPM monomers and oligomers and for estimation of their electrophoretic mobility. The monomer/oligomer band intensity ratio in semi-native conditions reflects the propensity of oligomers to dissociate into the monomers. Results are shown in Fig. 2a. Whereas C21A exhibited a high-MW band (presumably oligomers) identical to that of NPMwt under native conditions, the band from C21F was located at the position corresponding to the weak lower-MW fraction of NPMwt or NPMmut (presumably monomers). In these experiments, the band from endogenous NPM oligomers served as a loading and position control. The results from semi-native electrophoresis in Fig. 2b show markedly increased monomer/oligomer ratio of C21A compared to NPMwt and absence of C21F oligomers. These results suggest that although NPM oligomerization seems to be unaffected by the C21 point mutation in living cells, the stability of the oligomers is considerably attenuated. Similar results were obtained with mRFP1-labeled variants. Interestingly, mRFP1-labeled proteins displayed slightly lower mobility in the native conditions. Moreover, R_NPM oligomers were found to be somewhat more stable compared to G_NPM ones (Fig. 2b).

We have not observed any changes in expression and in oligomerization state of the endogenous NPMwt in cells containing C21 mutants. Therefore, we further investigated the effect of C21F substitution on the stability of exogenous heterooligomers formed by a mixture of fluorescently labeled C21F and NPMwt. 293T cells were alternatively transfected with single fluorescent variants of C21F and NPMwt, or co-transfected with the combination of both. As shown in Fig. 3 and Supplementary Fig. S1, the exogenous NPMwt monomer/oligomer ratio is strongly affected by the presence of C21F. Band intensities attributed to C21F oligomers and NPMwt monomers are both considerably higher in traits corresponding to the co-transfected sample than the ones in traits from single transfected cells. Distribution of NPM molecules between oligomer (high-MW) and monomer (low-MW) bands likely depends on the NPMwt/C21F participation in the heterooligomers. Interestingly, endogenous NPM monomer/oligomer ratio seems to be unaffected by the presence of any exogenous NPM. Altogether, despite the C21F mutation does not completely abrogate NPM oligomerization, it clearly attenuates interaction affinities underlying the mixed oligomer formation in living cells.

Localization and oligomerization properties of NPM N-terminal deletion mutants. In view of the fact that the C21-point mutations do not cause any detectable changes of the NPM oligomerization in living

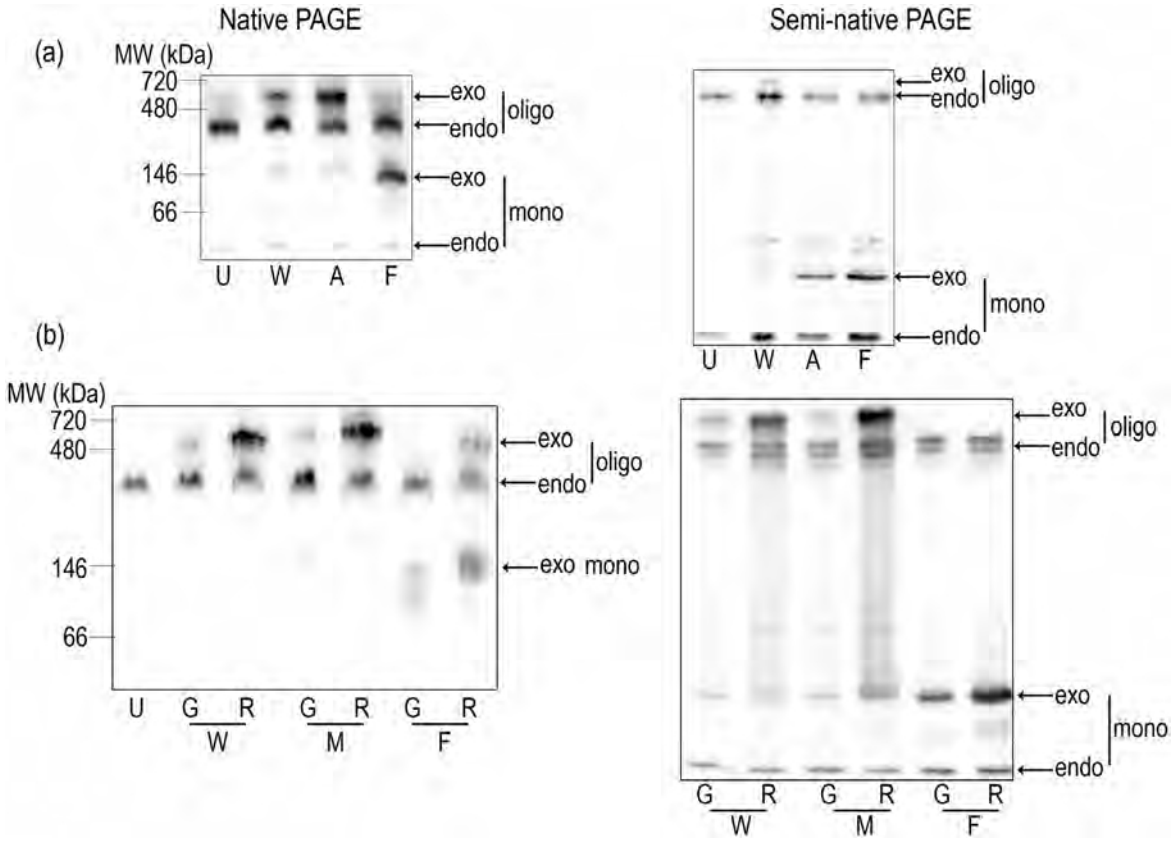

Figure 2. Effect of C21 mutations on the stability of NPM oligomers. Native (left) and semi-native (right) PAGE analysis of lysates from untransfected (U) 293T cells or cells transfected with NPMwt (W), C21A (A), C21F (F) or NPMmut (M). (**a**) Stability of oligomers formed by various G_C21 constructs is affected by the reducing PAGE conditions. (**b**) Position of bands and oligomer stability depend on the fluorescent label used (G or R: eGFP- or mRFP1-labeled proteins). The figures show representative examples from repeated experiments.

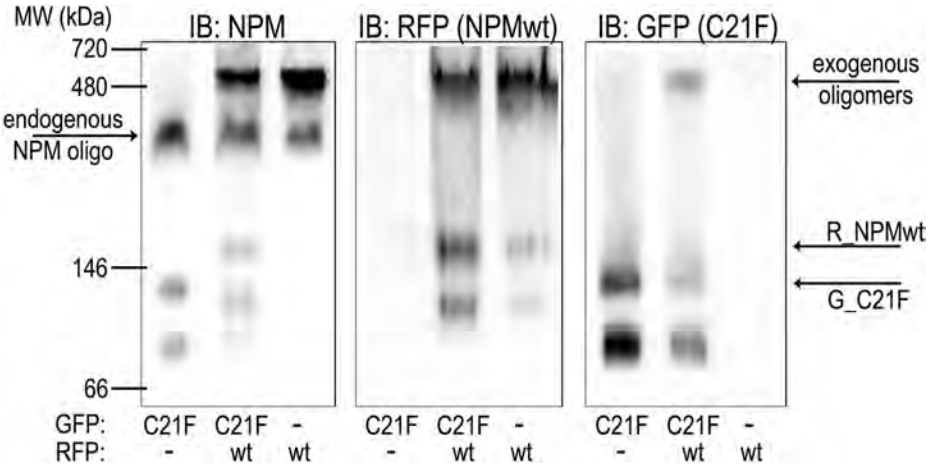

Figure 3. Formation of heterooligomers containing NPM with C21F substitution and NPMwt. Western blots of native PAGE of samples from 293T cells transfected with R_NPMwt (wt), G_C21F (C21F), and with their combination. Similar results were obtained with the inverse tagging, i.e. with G_NPMwt and R_C21F.

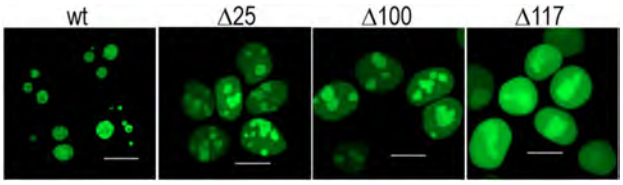

Figure 4. Significance of the N-terminus for NPM localization. Diminished nucleolar accumulation and increased amount in the nucleoplasm of Δ25, Δ100 and Δ117 compared to WT.

cells, we searched for other modifications of the NPM oligomerization domain in order to validate detection methods for the oligomerization disruption. Numerous NPM N-terminal deletion mutants were reported to lose the oligomerization ability and the nucleolar localization depending on the extent and specificity of the deleted region[56]. Enomoto et al.[56] determined residues and regions accountable for NPM oligomerization, for its nucleolar localization, and for p14Arf binding. Their data disclosed that NPM lacking a part of the N-terminal domain was localized in the nucleoplasm and exhibited inability to interact with other NPM molecules. For negative controls in living cells, we therefore created fluorescently labeled N-terminal NPM mutants with deletions of the first 25, 100 and 117 amino acids (Δ25, Δ100, Δ117) and we analyzed their subcellular localization and oligomerization characteristics. Our confocal imaging experiments have revealed that all the truncated NPM forms reside both in the nucleoli and in the nucleoplasm (Fig. 4). As expected, the largest deletion resulted in an increased accumulation of the mutant in the nucleoplasm.

To monitor NPM complex formation, lysates from 293T cells transfected with fluorescently labeled deletion mutants were subjected to electrophoresis. As expected, inability of the truncated proteins to form oligomers was reflected by an absence of the high-MW bands under native and semi-native conditions (Fig. 5a). Further we searched for presence of NPM heterooligomers containing exogenous NPMwt and selected deletion mutants. 293T cells were co-transfected with plasmids ensuring expression of Δ117 and NPMwt and the lysates were analyzed (Fig. 5b and Supplementary Fig. S2). It can be seen that bands from oligomeric NPMwt complexes are unaffected by the presence of Δ117 in the native as well as in the semi-native immunoblots. The result suggests that, in contrast to the C21 mutants, the presence of the deletion mutants does not affect the oligomerization of NPMwt. Again, expression of the endogenous NPMwt remained unchanged. To analyze the interaction potential of the N-terminal deletion mutants, 293T cells were transfected with plasmids encoding for GFP-labeled NPMwt, Δ25, Δ100 or Δ117. Then immunoprecipitation using GFP-Trap was performed and the precipitates were analyzed for the presence of endogenous NPM as well as for presence of other nucleolar proteins known to interact with NPM. Surprisingly, although the GFP-tagged deletion mutants displayed no ability to oligomerize, interaction of the endogenous NPM with any deletion mutant (except the plasmid encoding for free eGFP) was detected. The amount of co-precipitated endo-NPM was only slightly lower compared to the G_NPMwt precipitates (Fig. 5c). Simultaneously, level of co-precipitated nucleolin (NCL), which does not interact with the AML-related NPMmut[49], was higher in precipitates of the truncated NPM forms. The level of another co-precipitated nucleolar protein, fibrillarin (FBL), also positively correlated with the extent of N-terminus deletion. On the other hand, the tumor suppressor p14Arf, which is known to interact with the N-terminal NPM domain[56], clearly co-precipitated only with the G_NPMwt.

To evaluate the utility of the method for detection of the complexes containing both NPMwt and the deletion mutants, we performed GFP/RFP-immunoprecipitation from cells co-transfected with R_NPMwt and G_Δ117. The co-transfection of R_NPMwt and G_C21F served as an interacting control. Whereas NPMwt was clearly detected in samples obtained by precipitation of the deletion mutant, the vice versa co-precipitation failed (Fig. 5d). Identical results were obtained for combination NPMwt+Δ100. No interaction was found between two color variants of the N-terminal mutant (Supplementary Fig. S3).

The Förster resonance energy transfer (FRET) is a robust spectroscopic method for evaluation of a donor–acceptor proximity and works as a "ruler" on the nanometer scale. Due to the inverse 6th-power dependence of the transfer efficiency on the donor–acceptor distance[57], the energy transfer occurs only between closely separated donor–acceptor pairs. This occurs e.g. within NPM oligomers comprising eGFP- and mRFP1-tagged subunits, where eGFP is a donor and mRFP1 acceptor. Complex formation therefore results in more efficient FRET, which is reflected in decreased fluorescence lifetime of eGFP. FRET-induced changes of the donor fluorescence lifetime can be mapped across the microscopic samples by fluorescence lifetime imaging (FLIM) [58]. FRET-FLIM is perfectly suited for live-cell imaging and we previously successfully used it to detect complexes formed by NPMwt and C21 mutants in living cells[55]. In the present work, the FRET between eGFP as a donor and mRFP1 as an acceptor attached to NPMwt and Δ117 (Δ100) was examined. Presence of FRET within the complex was detected by the mRFP1 photobleaching, since an increase in the eGFP lifetime upon the mRFP1 photodestruction is a strong positive indicator of the mixed-complex formation. Results are shown in Fig. 6. Panels A and B show the initial localization of the green and red signal within the cells, panels C and D the intensity ratio I_{red}/I_{green} before and after the photobleaching, respectively. Corresponding FLIM images and lifetime histograms from the analyzed nucleolar area are shown in the panels E, F and G, respectively. As expected, a significant lifetime increase resulting from the FRET cancellation was detected after photobleaching in cells co-transfected with G_NPMwt and R_NPMwt. On the contrary, virtually no effect was observed in cells transfected with the G_Δ117 + R_Δ117 mutants. In accord with the literature[56] and with our precipitation data, this result

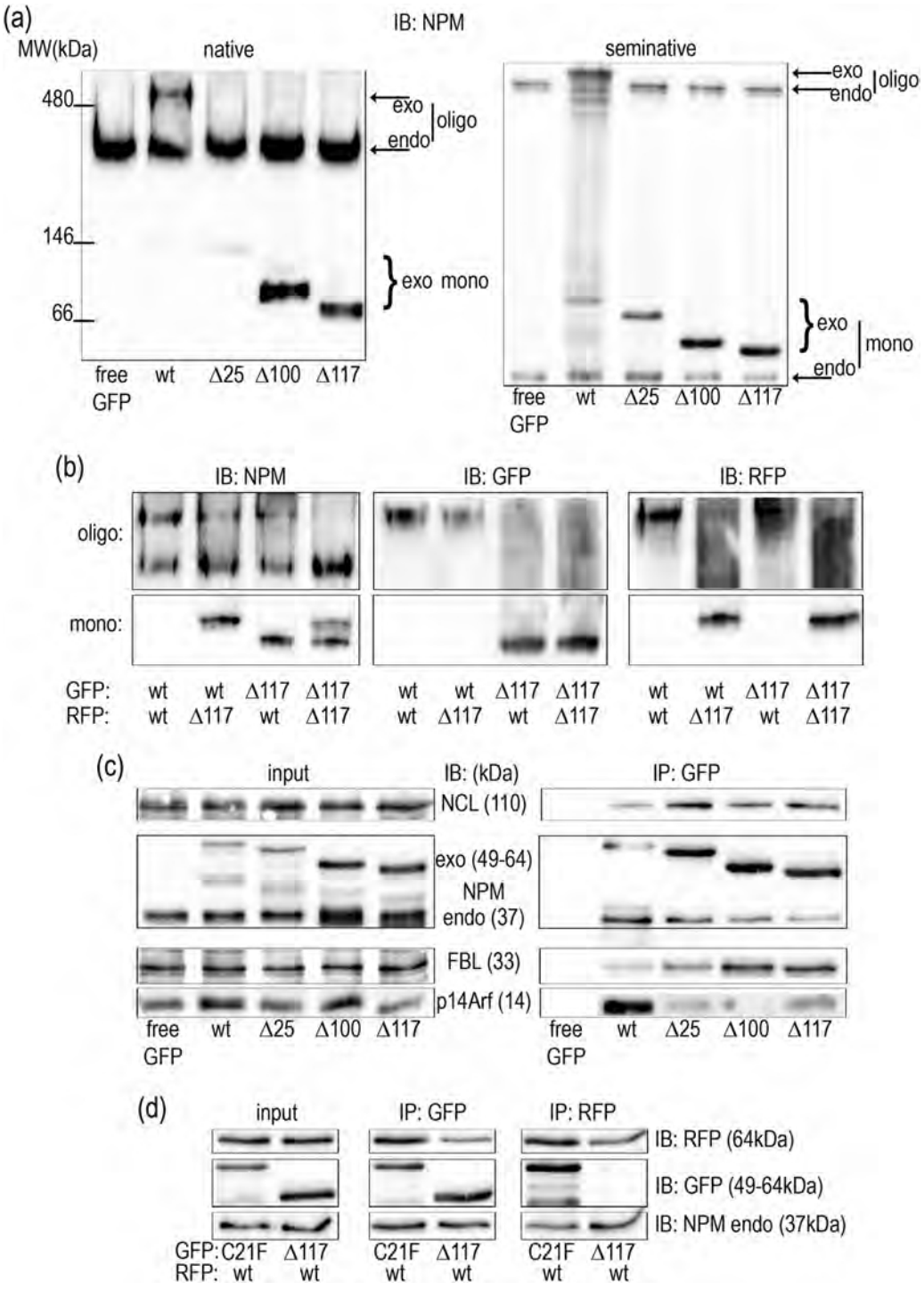

Figure 5. Significance of the N-terminus for NPM oligomerization. (**a**) NPM expression in native and semi-native PAGE of 293T cells transfected with free eGFP and eGFP-tagged truncated NPM variants. (**b**) Native PAGE of 293T cells co-transfected with combinations of Δ117 and NPMwt illustrating absence of interaction between Δ117 and NPMwt. (**c**) Interaction of truncated NPM forms with endogenous proteins. Lysates from 293T cells expressing GFP-labeled NPMwt, Δ25, Δ100 and Δ117 were subjected to immunoprecipitation and the levels of co-precipitated interaction partners were investigated. (**d**) eGFP/mRFP1-immunoprecipitation from 293T cells co-transfected with R_NPMwt and G_C21F or G_Δ117: asymmetric results of precipitation from the sample containing the truncated NPM form.

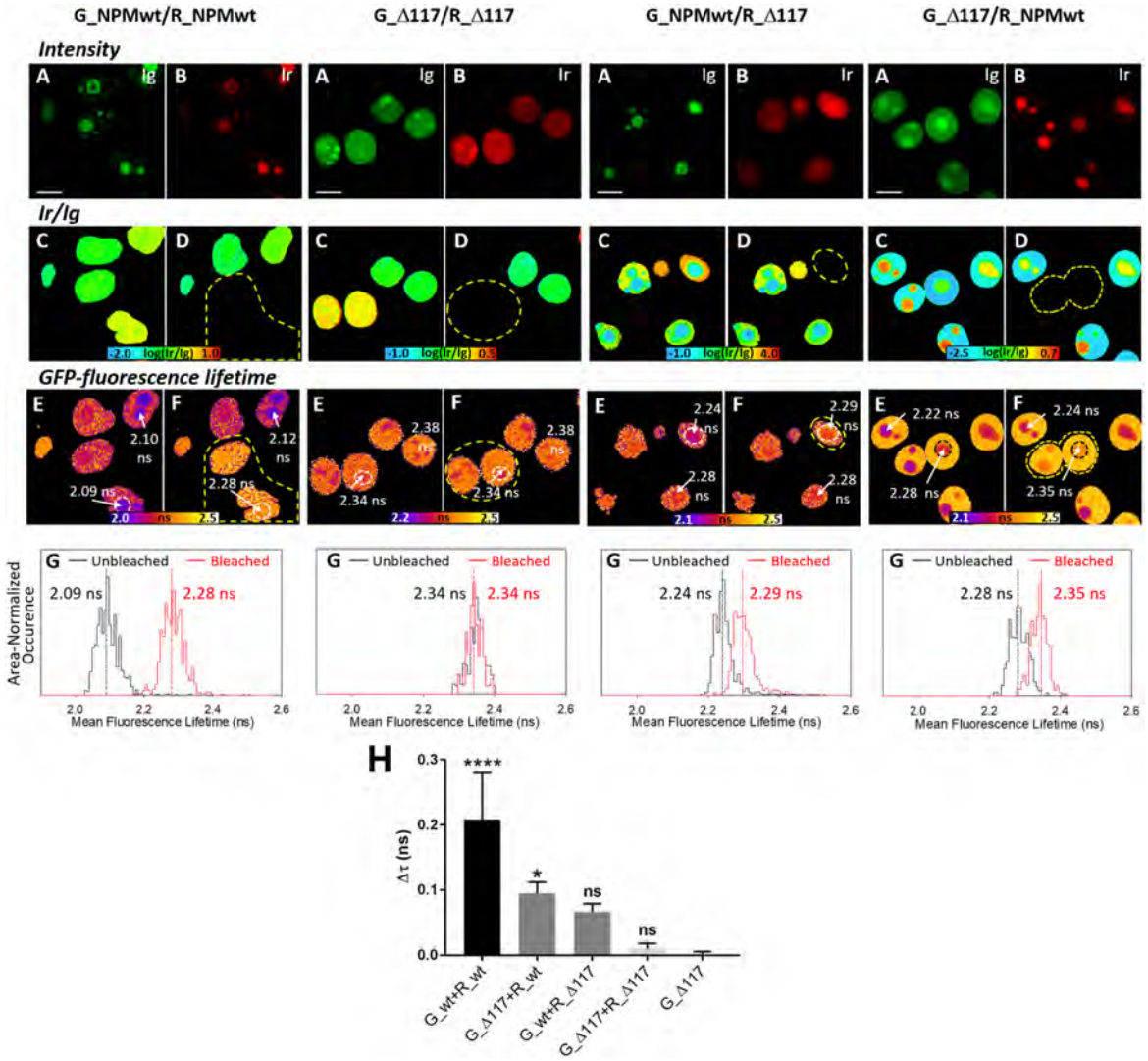

Figure 6. Effects of deletion in the N-terminal domain of NPM on its oligomerization in living 293T cells studied by FLIM-FRET. Interaction of eGFP- and mRFP1-labeled NPMwt causes shortening of the eGFP-fluorescence lifetime (τ) in co-transfected cells. After mRFP1-photobleaching, τ becomes prolonged, which confirms the G_NPMwt/R_NPMwt interaction. No lifetime change after mRFP1 photobleaching suggests absence of interaction between G_Δ117 and R_Δ117. (**A**,**B**) Initial localization of the green and red signal within the cells; (**C**,**D**) The intensity ratio of I_{red}/I_{green} before and after the photobleaching, respectively. (**E**,**F**) Fluorescence lifetime distribution of eGFP before and after the mRFP1-photobleaching; (**G**) lifetime histograms from the analyzed nucleolar area; (**H**) One-way ANOVA analysis of the eGFP fluorescence lifetime change after acceptor photobleaching. Changes are compared to the negative control G_Δ117. Error bars represent ± SD of at least 3 independent experiments (****$p < 0.0001$; *$p < 0.05$).

indicates inability of the Δ117 mutants to interact with each other and to form multimers. This protein pair can therefore serve as a negative control for the multimer formation in the NSC348884 experiments. As seen from Fig. 6, in samples containing combination of the Δ117 deletion mutants with NPMwt, the GFP-lifetime slightly, but still visibly, increases upon the acceptor photobleaching. The change corresponds with the results of our electrophoretic experiments and suggests some amount of the mixed multimer to be present in the cell. The statistical analysis from multiple experiments (n = 3–5) is presented in Fig. 6H, where cells transfected with G_Δ117 (the donor only transfection) served as a negative control. The figure clearly proves oligomerization of NPMwt. Oligomerization of Δ117 deletion mutants is clearly undetected and the presence of mixed NPMwt + Δ117 multimers is at the significance limit. Interestingly, the statistical evaluation also suggests the asymmetric character of interaction between NPMwt and Δ variants. These results are in a nice agreement with the immunoprecipitation.

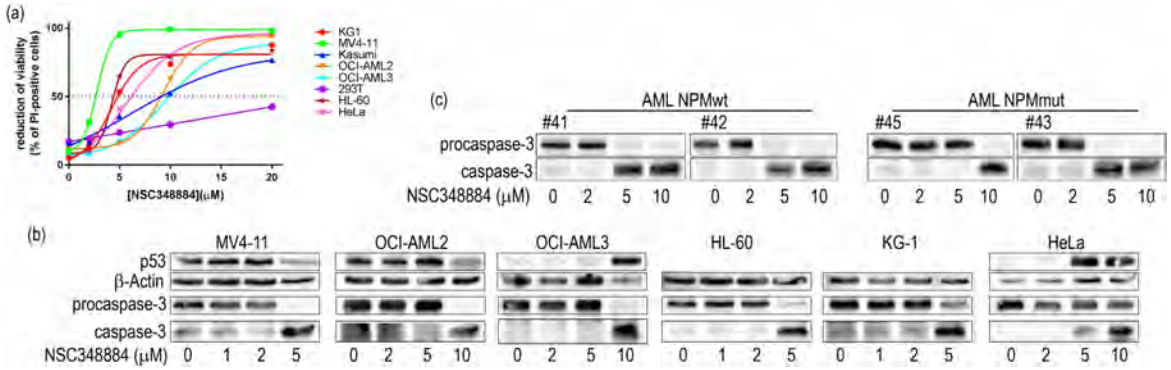

Figure 7. The effect of 24 h NSC348884-treatment on cell viability and apoptosis. (**a**) Cell viability monitored by propidium iodide exclusion: each point represents the mean value of 3–10 independent experiments. (**b**,**c**) Representative blots of caspase-3 fragmentation and p53 expression in cell lines (**b**) and in primary AML cells (**c**). β-Actin levels serve as a loading control.

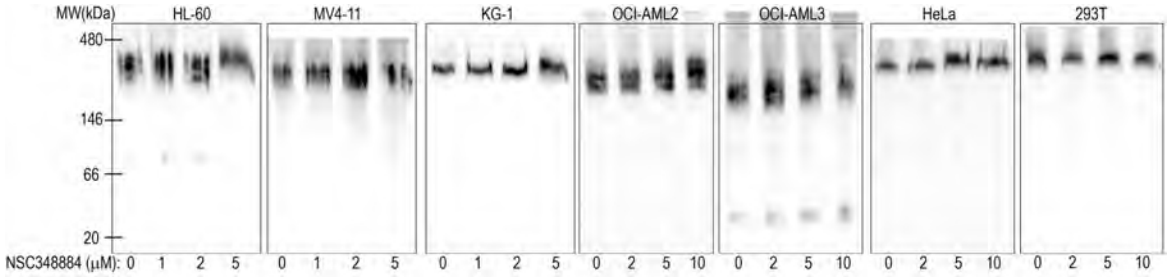

Figure 8. Effect of NSC348884 treatment on NPM oligomerization in leukemia cells. Native PAGE, representative blots: effect of 24 h NSC348884 treatment on NPM oligomerization in various leukemic cell lines as well as in adherent HeLa and 293T cells.

Effect of NSC348884 on cell viability, apoptosis and NPM oligomerization. The small molecule NSC348884 was reported to interfere with NPM oligomerization in solid tumor cell lines[50] as well as in leukemia cells[41]. Furthermore, it was proved to inhibit proliferation, to upregulate p53 and to trigger apoptosis[41,50]. We thus investigated the influence of NSC348884 treatment in a panel of leukemia cell lines complemented with HeLa and 293T cells.

Prior to characterization of the NSC348884 effect on NPM oligomerization, we performed the analysis of cell viability and apoptotic markers. The cell viability in the presence of NSC348884 was monitored by propidium iodide (PI) exclusion test (Fig. 7a). Caspase-3 fragmentation as well as changes of p53 expression were investigated by immunoblotting to assess the extent of apoptosis in NSC348884-treated cells (Fig. 7b). For the majority of the cell lines, the EC50 value was within the interval from 2 to 10 μM. The viability drop correlated with increased caspase-3 fragmentation indicating the onset of apoptosis (Fig. 7b). Simultaneously, NSC348884-induced increase in the p53 level was detected in some of the cell lines possessing wild-type p53. Contrarily to previously reported results[41], the majority of cell lines with NPMwt was more sensitive than the cell line with NPMmut (OCI-AML3). Comparable sensitivity (from caspase-3 fragmentation) to NSC348884 treatment was found also for the primary cells of AML patients regardless of their NPM mutational status (Fig. 7c). Unexpectedly, native PAGE experiments revealed no influence of NSC348884 on NPM oligomerization (Fig. 8). Endogenous NPM oligomers were found to be stable in KG-1, HL-60, MV4-11, and HeLa cell lines, which exhibited extensive apoptosis after NSC348884 treatment, as well as in OCI-AML2, OCI-AML3 and 293T, which were substantially more resistant to the treatment.

To further investigate effect of NSC348884 in vivo, we co-transfected 293T and HeLa cells with R_NPMwt and G_NPMmut. Then the cytoplasmic localization of R_NPMwt was monitored for 2 h after addition of 10 μM NSC348884 (Fig. 9). In agreement with our previous results[36,59], detectable fraction of R_NPMwt was found in the cytoplasm of both cell lines at the starting time point. Lower cytoplasmic fraction of R_NPMwt in HeLa cells (compared to 293T) likely results from a higher endogenous NPM level[36,60]. Importantly, the cytoplasmic localization of R_NPMwt remained unchanged for at least 2 h after the treatment suggesting independence of NPM oligomerization on the presence of NSC348884 in vivo (Fig. 9). Simultaneously, there was an obvious effect of NSC348884 on cell-surface adhesivity. The effect is clearly visible in transmitted light images (DIC). Whereas the 293T cells progressively rounded and finally lost their contact with the glass surface of the culture dish, the

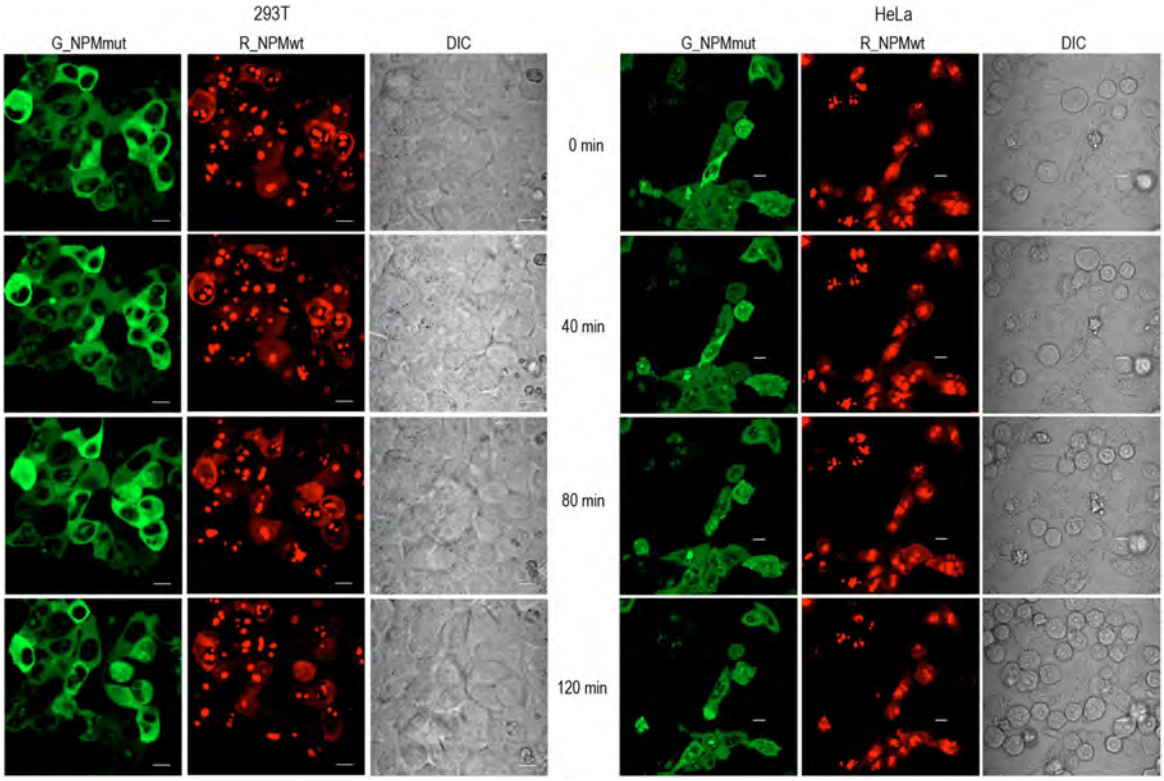

Figure 9. Time course of NSC348884-induced effects on the cell morphology. Effect of 10 μM NSC348884 was monitored in cells co-transfected with R_NPMwt (red) and G_NPMmut (green). The presence of the red signal in the cytoplasm and the cell morphology (DIC) were analyzed under confocal microscope. Left: 293T cells; right: HeLa cells. Scale bar represents 10 μm.

HeLa cells detached from the surface individually. In any case, mitotic cells that detached from the surface for cell division have never re-adhered.

The oligomerization of fluorescently labeled NPM was finally tested by the native PAGE and by immunoprecipitation in cell lysates and by FRET in living cells. First, we tested whether the low-MW band attributed to NPM monomers appears in native lysates of 293T cells expressing a combination of G_NPMmut and R_NPMwt after the NSC348884 treatment. Lysate from cells co-expressing G_NPMmut and weakly oligomerizing R_C21F was used to mark the position of the low-MW band (Fig. 10a and Supplementary Fig. S4). No difference between the control and the NSC348884-treated sample was found either under native or semi-native conditions. Similar results were obtained from cells co-transfected with alternative combinations, i.e. with G_NPMwt + R_NPMwt or with G_NPMmut + R_NPMmut (Fig. 10b). Identical samples were afterwards subjected to immunoprecipitation (GFP- and RFP-Trap). All the exogenous NPM forms as well as the endogenous NPM were detected in all GFP- and RFP-precipitates regardless the NSC348884 addition (Fig. 11). In agreement with our previous work[49], control experiment revealed that NCL co-precipitated with NPMwt and it did not co-precipitate with any form of NPMmut. Again, the NPM-NCL interaction was not affected by the presence of NSC348884 in any experiment.

Oligomerization in living cells was independently tested by the resonance energy transfer. As seen from Fig. 12, FLIM-FRET experiments reveal unchanged eGFP fluorescence lifetime upon NSC348884 treatment of cells co-transfected with donor- and acceptor-labeled NPMwt and NPMmut. Prolonged eGFP-fluorescence lifetime after mRFP1-photobleaching confirmed the complex formation in control cells without NSC348884 (column 1 and 2). The NSC348884-treatment did not affect the lifetime pattern (column 3) and the second round of the acceptor bleaching confirmed persistence of heterooligomers despite the presence of NSC348884 (column 4). Lower FRET extent in the NPMmut co-transfected cells (the second row) is likely a result of lower cytoplasmic NPMmut concentrations and consequent lower complex formation. Nevertheless, presence of FRET is still detected. NSC348884 activity resulting in cell rounding and loss of their contact with the glass surface is visibly documented by the morphology screening during the FLIM experiments (columns 5, 6), similarly to Fig. 9. No lifetime change following mRFP1-photobleaching or NSC348884 treatment was detected in the control sample (Supplementary Fig. S5), i.e. in cells expressing two color variants of NCL, where FRET should not be detected[55]. Altogether, FLIM-FRET experiments confirmed that NSC348884 does not affect NPM oligomerization, although it influences apoptosis and cell adhesion.

To further investigate the mechanism of NSC348884 action, we analyzed changes in the cell-surface contact after NSC348884 addition with help of Electric Cell-Substrate Impedance Sensing (ECIS) technique. ECIS allows

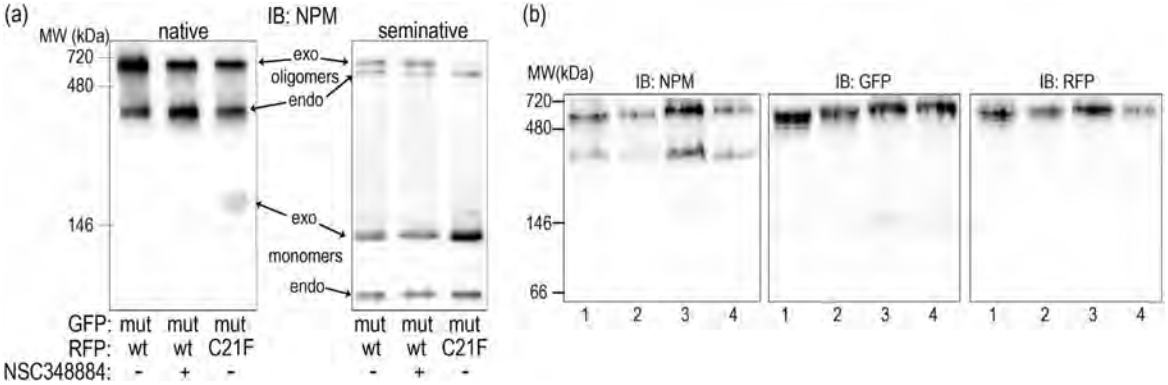

Figure 10. Native and semi-native PAGE from 293T cells transfected with various fluorescent variants of NPM and treated with 10 μM NSC348884 for 24 h. (**a**) Cells co-transfected with G_NPMmut and R_NPMwt (lanes 1 and 2) or R_C21F (lane 3). Endogenous NPM, R_NPMwt and G_NPMmut oligomers detected in untreated (lanes 1 and 3) and NSC348884-treated (lane 2) cells. (**b**) Native PAGE, G_NPMwt + R_NPMwt (1, 2) and G_NPMmut + R_NPMmut (3, 4) in control (1, 3) and NSC348884-treated (2, 4) cells.

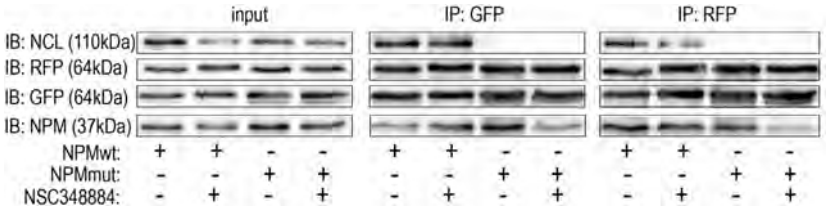

Figure 11. Interaction potential of NPM in NSC348884-treated cells. Immunoprecipitation from 293T cells co-transfected with G_NPMwt + R_NPMwt (NPMwt) or G_NPMmut + R_NPMmut (NPMmut) in control and NSC348884-treated sample (10 μM NSC348884 for 24 h). All exogenous forms as well as the endogenous NPM were detected in all eGFP- and mRFP1-precipitates regardless the NSC348884 addition. Nucleolin (NCL) was detected in NPMwt-precipitates only, independently of the NSC348884 treatment.

for non-invasive real-time monitoring of cell interaction with the surface of the sample well. Small microelectrodes embedded in the bottom of the ECIS plate serve to measure the impedance in a range of frequencies of the sensing electric current, and the signal is then automatically decomposed into resistance and capacitance. In our experiments, the capacitance component at a high frequency (64 kHz) mirrored that of the resistance at 2 kHz and reflected mainly the area of the cell-surface contact. Before the drug addition, the progressive increase of the signal reflects cell attachment and proliferation. With this device, we were able to follow the time course of the adhesivity decrease after NSC348884 addition (Fig. 13). We have reported previously, that inhibition of SRC family kinases by dasatinib resulted in a rapid drop of ECIS signal, which corresponded to cell shrinkage, and we thus used dasatinib as a reference compound[61]. In both adherent cell lines, 293T and HeLa, NSC348884 induced large, dose-dependent changes in the resistance signal, which were similar to those produced by IPA-3[62], an inhibitor of p21-activated kinases (PAK). PAK are key regulators of adhesion signaling, which have been proposed as therapeutic targets in different kinds of cancer including leukemias[63,64]. We thus analyzed possible effect of NSC348884 on expression and activity of PAK1, as well as of Cofilin, which governs actin remodeling during changes of cell shape. Indeed, Ser144 phosphorylation of PAK1 reporting on its kinase activity was reduced after 2 h of NSC348884 treatment whereas total PAK1 expression remained unchanged (Fig. 14). Simultaneously, inactivating phosphorylation at Ser3 of Cofilin was detected.

Discussion

The N-terminal region of NPM is essential for its oligomerization as well as for its chaperone function as numerous proteins interact with NPM through this domain. Since AML-related NPM mutation does not substantially affect its ability to form oligomers, NPM-interacting proteins become frequently mislocalized together with aberrantly localized NPMmut. Targeting the NPM oligomerization offers a possibility to manipulate localization of the interacting partners. Simultaneously, profiting from slightly different oligomerization properties of NPMmut and NPMwt, a fine control of NPM oligomerization by appropriate concentrations of oligomerization-inhibiting drugs might have a therapeutic effect in the AML with *NPM1* mutation. Although several alterations of NPM N-terminal domain were reported to disrupt NPM oligomerization in vitro, results thoroughly describing NPMwt and NPMmut oligomerization in vivo are missing. We have previously documented that C21 point mutations

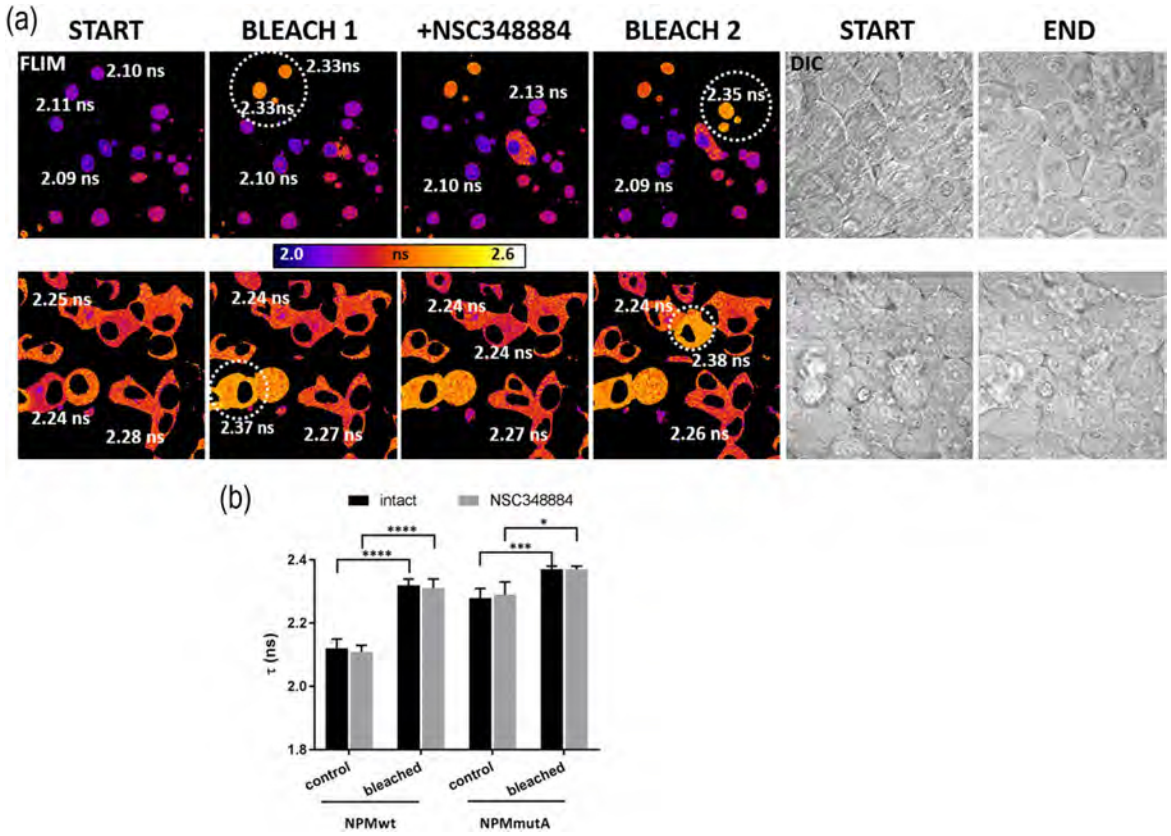

Figure 12. NPM oligomerization after NSC348884 treatment in living cells. (**a**) FLIM-FRET analysis of the eGFP-fluorescence lifetime (τ) after 2 h of 10 μM NSC348884 action on cells co-transfected with red and green variants of NPMwt (upper row) or NPMmut (lower row). White numbers: fluorescence lifetime measured in individual cells. Dashed circles: region of mRFP1-photobleaching. Simultaneous cell morphology screening by DIC documents cell rounding induced by NSC348884. (**b**) Statistical evaluation of τ values before (control) and after (bleached) the mRFP1 photobleaching in intact (black bar) and NSC348884-treated (grey bar) cells. Student´s t-test of "control" versus "bleached" values: $^{****}p < 0.0001$, $^{***}p < 0.001$,$^{*}p < 0.05$.

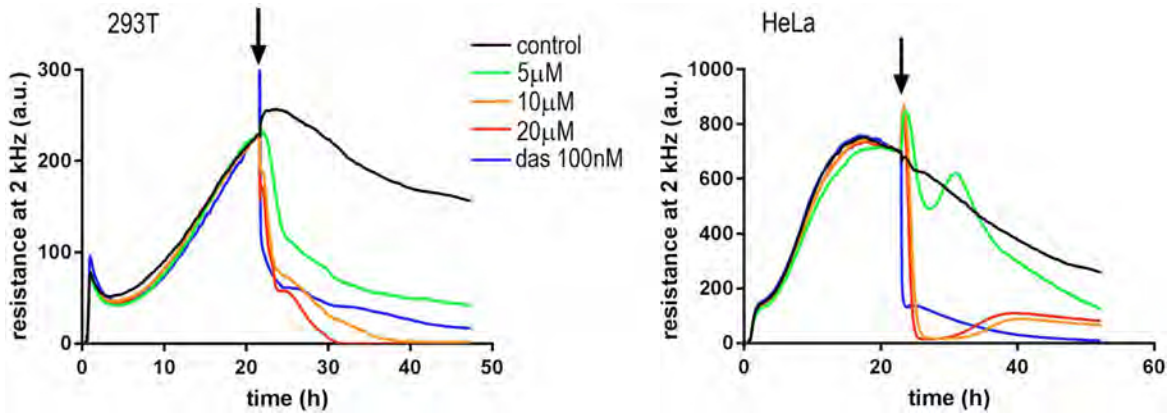

Figure 13. Decrease of the cell-surface contact area after NSC348884 addition. Resistance at 2 kHz was tracked during 293T (left) or HeLa (right) adhesion to the well bottom and after NSC348884 or dasatinib (das) addition. Times of the drug addition are marked by arrows. The curves represent mean values of triplicate/duplicate wells for NSC348884/das, respectively.

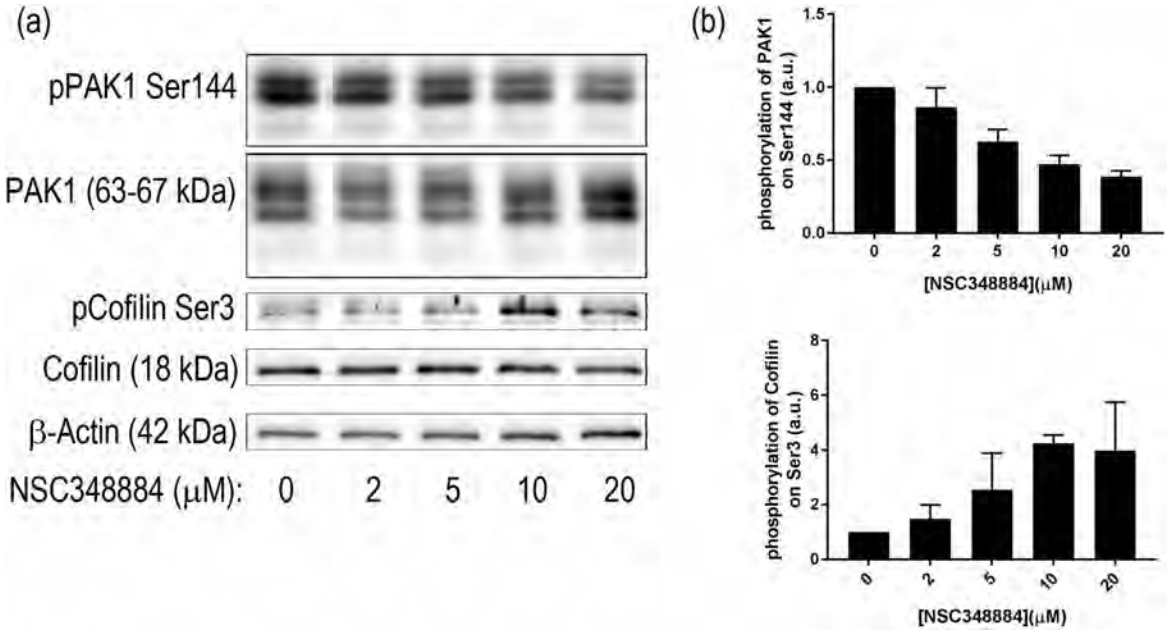

Figure 14. Expression and phosphorylation of adhesion-related protein kinase PAK1 and of the actin regulator Cofilin in 293T cells after 2 h NSC348884 treatment. (**a**) Representative blots, (**b**) summary from 2 experiments. Error bars: ± SD.

do not disrupt NPM oligomerization in living cells[55]. Here we demonstrate that the cytoplasmic mislocalization of R_C21 and R_NPMwt in cells co-expressing G_NPMmut is very similar (Fig. 1). This strongly indicates an existence of interaction between natural NPM forms and the C21 mutants. Our results from native and semi-native electrophoreses allowed us to evaluate the aggregation potential of C21 mutants depending on the substituting aminoacid (Fig. 2) in vitro, in agreement with the results of Prinos et al.[54] obtained mainly in experiments with recombinant proteins. Whereas C21F substitution resulted in disruption of NPM oligomers under native conditions (Fig. 3 and Supplementary Fig. S1), the effect of the C21A substitution was only detectable under reducing conditions. NPM oligomers were reported to consist of five NPM molecules[30], and formation of heterooligomers containing NPMwt and NPM mutants were found to be highly frequent[35,36]. We therefore suggest that the in vivo stability of NPM heterooligomers is permitted by a sufficient number of NPMwt molecules in the heterooligomeric complex. Thus, in living cells, the C21 point mutations do not have potential to fully disrupt these complexes, although their ability to retain in oligomers is compromised under in vitro conditions.

In agreement with results of Enomoto et al.[56], partial or complete deletion of NPM oligomerization domain (aa1-117) led to delocalization of the truncated protein from the nucleoli to the nucleoplasm (Fig. 4). However, even the NPM with completely deleted N-domain (Δ117) exhibited higher concentration in the nucleoli compared to the nucleoplasm. This is likely due to the fact, that nucleolar localization signal as well as nucleic acid binding domains remain unaffected by the deletion. We have found that oligomerization of the deletion mutants was completely abrogated and no interaction between two truncated NPM forms was detected (Fig. 5 and Supplementary Figs. S2, S3). Nevertheless, immunoprecipitation revealed presence of both exo- and endogenous NPMwt in the G_Δ117 precipitates indicating that the truncated NPM yet participates in complexes that are possibly too large to enter the native gel. The existence of mixed Δ117/NPMwt complexes is also supported by the FRET results monitoring the Δ117-NPMwt interaction in living cells (Fig. 6). Enhanced level of nucleolar proteins NCL and FBL co-precipitated with the deletion mutants suggests better accessibility of the NPM region responsible for binding of these proteins. Both NCL and FBL were previously found to reside in the nucleoli of cells with NPM mutation[46,49,59] and they may therefore mediate the nucleolar localization of the deletion mutants. Complexes containing NCL and/or FBL together with NPMwt and Δ117mutants thus represent a potential pool of proteins that can co-precipitate with the deletion mutants.

NSC348884 is declared to inhibit NPM oligomerization[50]. Its structure was obtained by in silico screening using a small molecular library. As the crystal structure of the human NPM was not available, *Xenopus* NO38-core chaperone structure (residues 1–107) was used for the screening. The sequence identity of this structure with equivalent part of human NPM oligomerization domain is 77%. NSC348884 was identified as the best candidate for inhibition of the dimerization interface of this polypeptide. Authors of the original paper[50] used native electrophoresis to demonstrate NSC348884-induced oligomer disruption in LNCaP and HCT116 cell lines. They detected diminished bands near 121 kDa, which they attributed to NPM oligomers. Surprisingly, the intensity of monomer bands remained unchanged. Other complex phenomena like induction of apoptosis and p53 upregulation were also found in NSC348884-treated cells[41,50]. From the available data it seems that function

of NSC348884 as an inhibitor of the full-length human NPM oligomerization was not unequivocally proven. We therefore analyzed effect of NSC348884 on various leukemia cell lines and on cells expressing fluorescently labeled NPM constructs. First, we tested cell viability and apoptotic signatures in order to determine the range of proper NSC348884 concentrations for the live-cell experiments. Concentrations required for a substantial viability decrease and caspase-3 fragmentation fell into the interval of 2–10 μM for the majority of the cell lines (Fig. 7). Neither the OCI-AML3 cell line nor the primary cells of AML patients with NPM mutation displayed enhanced sensitivity to NSC348884 treatment. Consistently with previous results[41,50], NSC348884 induced p53 upregulation in some cell lines. We also noticed NSC34884-induced downregulation of p14Arf in 293T and HeLa cells (data not shown). In contrast to results of Balusu et al[41], none of the tested cell lines nor primary cells from AML patients displayed any change in the NPM oligomerization upon treatment with efficient NSC348884 concentrations when investigated by the native and semi-native electrophoreses (Fig. 8). Similarly, oligomers containing fluorescently labeled NPMwt and NPMmut were not affected by the NSC348884 treatment (Fig. 10 and Supplementary Fig. S4). These results were further verified by immunoprecipitation where both exogenous and endogenous NPM co-precipitated with both GFP- and RFP-labeled NPMwt and NPMmut, despite the presence of NSC348884 (Fig. 11). Also the fluorescence microscopy revealed sustained fraction of NPMwt in the cytoplasm of NSC348884 treated cells co-expressing NPMwt and NPMmut, which witnesses for their interacton. Accordingly, the FLIM-FRET proved persisting interaction between fluorescently labeled NPM molecules upon the NSC348884 treatment (Fig. 12). Cells expressing two fluorescent variants of likely noninteracting NCL were used as a control (Supplementary Fig. S5). As expected, NCL molecules labeled with the eGFP donor and the mRFP1 acceptor on their N-termini did not exhibit any FRET, which was proved by a zero lifetime change upon the acceptor photobleaching. The result was independent of the NSC348884 treatment. Compared to NCL, the presence of FRET in the cells with fluorescently labeled NPM is clearly detectable both before and after the NSC348884 treatment. We conclude that contrary to the published data[50], NSC348884 does not act as an oligomerization inhibitor and does not affect formation of NPM oligomers under physiological conditions. This finding is extremely important in view of the fact that this drug has been recently reported to cause numerous cellular effects, which were ascribed, in accordance with its declared function, to disruption of NPM oligomerization[51–53].

During the live-cell experiments, we noticed apparent changes in cell adhesivity. The cell-surface contact area during the NSC348884 treatment was therefore monitored by Electrical Cell-Substrate Impedance Sensing (ECIS) (Fig. 13). The rapid onset of changes in the ECIS signal indicated that the cell shrinkage and detachment was not a secondary effect accompanying apoptosis. As the course of the ECIS signal was similar to that induced by the inhibitor of p21-activated kinases, IPA-3[62], we investigated also activity and expression of PAK1 and Cofilin, a known actin regulator[63] (Fig. 14). The observed changes of both PAK1 and cofilin phosphorylation indicate that NSC348884 interferes with adhesion signaling. Further research is required to elucidate mechanistic role of NSC348884 in this process and its potential for anticancer therapy.

Conclusion

We have shown that a proposed inhibitor of NPM oligomerization, NSC348884, does not affect NPM oligomer formation in any of the examined leukemia cells. Moreover, the cell sensitivity to NSC348884 treatment is not potentiated by AML-associated NPM mutation. On the other hand, we have uncovered so far unknown effect of NSC48884 on the cell-surface adhesion, which could play a key role in the complex cellular response to the NSC48884 treatment.

In addition, our findings prove that point mutations in Cysteine 21 slightly potentiate oligomer dissociation but the overall NPM interaction potential with other NPM molecules remains conserved in living cells. Deletion mutants lacking part of the NPM N-terminal domain completely lose their oligomerization ability, but they partially retain the interaction with NPMwt, possibly through enhanced interaction with other nucleolar proteins in complexes with NPMwt.

Material and methods

All methods were carried according to Declaration of Helsinki.

Cell culture and chemicals. Leukemia cell lines MV4-11, OCI-AML2, OCI-AML3, KG-1, and KASUMI-1 were purchased from DSMZ (Germany), HL-60 were from ECACC (GB), adherent cell lines HEK-293T and HeLa were kindly provided by dr. Š. Němečková (Department of Immunology, Institute of Hematology and Blood Transfusion) and dr. J. Malínský (Institute of Experimental Medicine, Czech Academy of Science), respectively. The cells were cultivated in growth media with fetal bovine serum (FBS), glutamine and antibiotics (all from Sigma-Aldrich) according to manufacturers recommendation: MV4-11, KG-1, HL-60, and HeLa in RPMI-1640/10% FBS, OCI-AML2 and OCI-AML3 in alpha-MEM/20% FBS, KASUMI-1 in RPMI-1640/20% FBS and HEK-293T in DMEM/10% FBS. Peripheral blood mononuclear cells (PBMC) originated from leukapheresis of hyperleukocytic AML patients. PBMC were separated using Histopaque 1077 (Sigma-Aldrich), washed with PBS and resuspended in RPMI-1640 with 10% FBS. The presence of C-terminal NPM mutation was detected by PCR and the mutation type was determined by sequencing[26] and confirmed by Western blotting and immunofluorescence using specific anti-NPMmut antibody[49]. All patients signed informed consent to the use of their biological material for research purposes in agreement with the Declaration of Helsinki. The study has been approved by the Ethics Committee of the Institute of Hematology and Blood Transfusion of the Czech Republic. All cells were cultivated in 5% CO_2 atmosphere at 37 °C. Stock solution of 10 mM NSC34884 was added to cell suspensions to final concentrations and times as indicated in the Results section.

Plasmid construction and transfection. As we described in detail previously[36,59], the gene for NPM was amplified from cDNA library (Jurkat cells, Origene) by PCR and inserted to vectors peGFP-C2 and pmRFP1-C2 (originally Clontech), designed for expression of protein chimeras with a fluorescent protein connected to the N-terminus of the target protein, by standard methods of molecular cloning. NPM mutants were constructed by PCR using extended primers targeting NPM1 sequence neighboring regions cut from the N-terminus or containing the mutated part of the exon 12 of the *NPM1* gene complemented with appropriate restriction sites[36]. After amplification in *E. coli*, the plasmids with subcloned genes were purified with PureYield Plasmid Miniprep System (Promega) and transfected into adherent cell lines using jetPRIME transfection reagent (Polyplus Transfection).

Cell lysis and western blotting. *Cell lysis* As described previously[49], cells were washed with PBS and lysed depending on the intended application. For direct use in SDS-PAGE, the cells were lysed in Laemmli sample buffer (SB, 50 mM Tris pH 6.8, 2% SDS, 100 mM DTT, 10% glycerol), boiled at 95 °C for 5 min, centrifuged at 200.000 g/4 °C for 4 h and the supernatant was stored at − 20 °C. For other applications, the cells were lysed in Lysis buffer (LB, 10 mM Tris/Cl pH7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, protease and phosphatase inhibitors) for 30 min/4 °C, centrifuged at 20.000 g/4 °C for 10 min and supernatant was mixed 1:1 with the appropriate buffer.

Native and semi-native PAGE Lysates were mixed with 2xnative buffer (NB, 50 mM Tris pH6.8, 10 mM DTT, 10% glycerol) and subjected without boiling to 7.5% AA Tris-glycin gel without SDS for native electrophoresis, or to the gel with SDS (2%) for semi-native electrophoresis.

Western blotting Five to ten microliters of each sample were subjected to native or SDS-PAGE and transferred into PVDF or nitrocelulose membrane (BioRad). Mouse monoclonal antibodies against β-actin, GFP, dsRed, NCL, FBL, NPM (clone 3F291 for NPMwt + mut detection, clone E3 for NPMwt detection), and p14Arf were from Santa Cruz Biotechnology. All mouse primary antibodies were used at a dilution 1:100–1:500. Rabbit polyclonal antibody against NPMmut (pab50321, Covalab) was used at 1:2000 dilution. Rabbit monoclonal anti-PAK1 (1:2000, Abcam) and anti-PAK1-pSer144 (1:20,000, Abcam) and rabbit polyclonal antibodies and anti-Cofilin and anti-Cofilin-pSer3 (Cell Signalling Technology) were used for adhesion-related protein detection. Anti-mouse and anti-rabbit HRP-conjugated secondary antibodies were purchased from Thermo Scientific and used at concentrations 1:10.000–1:50.000. ECL Plus Western Blotting Detection System (GE Healthcare) was used for chemiluminescence visualization and evaluation by G-box iChemi XT4 digital imaging device (Syngene Europe). Alternatively, Alexa488-conjugated anti-rabbit and Alexa647-conjugated anti-mouse secondary antibodies (ThermoFisher) for simultaneous detection of NPMmut and NPMwt + mut were used.

Immunoprecipitation. Immunoprecipitation using GFP- or RFP-Trap (Chromotek) was performed according to manufacturer´s instruction as described previously[36]. Briefly, cells were harvested and washed with PBS, lysed in LB for 30 min/4 °C and centrifuged at 20.000 g/4 °C for 10 min. The lysate was mixed with GFP/RFP-nanobeads and rotated for 1 h/4 °C. The beads were extensively washed with diluting buffer (10 mM Tris/Cl pH7.5, 150 mM NaCl, 0.5 mM EDTA), resuspended in SB, boiled at 95 °C for 10 min and centrifuged 20.000 g/4 °C for 10 min. Supernatant was stored at − 20 °C until used for SDS-PAGE.

Live-cell imaging. The cells were seeded in the 2.5 mm culture dish with glass bottom (Cellvis) for 24 h and then transfected with plasmids containing fluorescent variants of the desired genes. After another 24 h, the transfected cells were observed under confocal laser scanning microscope FluoView FV1000 (Olympus Corporation) using 543 nm excitation for RFP fluorescence and 488 nm excitation for GFP and for differential interference contrast (DIC) observation. UPlanSAPO 60 × NA1.35 oil-immersion objective was used for imaging. For long-term monitoring, the culture dish was sealed by parafilm to prevent CO_2 leakage and it was placed into microscopy chamber tempered to 37 °C. NSC348884 was added just before the start of the measurement. Fluorescence images were processed by the FluoView software FV10-ASW 3.1.

Lifetime imaging and acceptor bleaching. The apparatus used for lifetime imaging is described in detail elsewhere[65]. Briefly, we used inverted IX83 microscope equipped with a FV1200 confocal scanner (Olympus, Germany), cell cultivation chamber (Okolab) and FLIM add-ons from PicoQuant. Fluorescence was excited by a pulsed diode laser (LDH-DC-485, 485 nm, PicoQuant) running at 20 MHz repetition rate. Light was coupled to the microscope by a single-mode optical fiber and reflected to the sample by 488 nm long-pass dichroic mirror (Olympus). Typically, UPLSAPO 60XW NA 1.2 water-immersion objective (Olympus) was used for imaging. Fluorescence was directed via multimode optical fiber to a cooled GaAsP hybrid PMT (PicoQuant) through the 520/35 bandpass filter (Semrock). Signal was processed by the TimeHarp 260-PICO TCSPC card and the SymPhoTime64 software (both PicoQuant). To avoid pile-up artifacts, the data collection rate at brightest pixels was kept below 5% of the excitation frequency. FLIM images were collected in a few minutes with the excitation power around 0.1 µW. Acceptor photobleaching was done by a 561 nm semiconductor CW laser with a multi-mW power at the focal point. All experiments were done at 37 °C.

Lifetime data processing. Lifetime images were generated in the SymPhoTime64 by the "fast-FLIM" method when pixel lifetimes were calculated by a method of moments[66]. Specifically, pixel lifetimes τ_{avg} were determined as the difference between the barycenter of the fluorescence decay and the time-offset t_{offset} of the steepest growth of the decay at each pixel:

$$\tau_{avg} = \sum I_i t_i / \sum I_i - t_{offset} \quad (1)$$

where I_i is a decay intensity at time t_i. Exported FLIM images were further processed and visualized by the Fiji software[67]. An accurate analysis of the cumulative decays from larger area of interest was done by the least-squares reconvolution also in the SymPhoTime64. Fluorescence was assumed to decay multiexponentially according to the formula:

$$I(t) = \sum_i \alpha_i \cdot \exp(-t/\tau_i 0), \quad \sum_i \alpha_i = 1 \quad (2)$$

where τ_i and α_i are the fluorescence lifetimes and the corresponding amplitudes, respectively. Typically, 2 decay components were sufficient for acceptable fit. The intensity-weighted mean fluorescence lifetime was calculated as:

$$\tau_{mean} = \sum_i f_i \tau_i = \sum_i \alpha_i \tau_i^2 / \sum_i \alpha_i \tau_i \quad (3)$$

where f_i are fractional intensities of the ith lifetime component:

$$f_i = \alpha_i \tau_i / \sum_i \alpha_i \tau_i, \quad \sum_i f_i = 1, \quad (4)$$

Electrical cell-substrate impedance sensing (ECIS). Impedance measurements were performed using the ECIS Zθ device (Applied Biophysics). The wells of 8W10E+ plates were filled with 200 µl culture medium and the baseline was monitored for several hours before cell addition. HeLa or 293T cells were seeded at 120.000 cells/well and monitored overnight, the inhibitors were added after 20–24 h. One well from each plate was left empty (medium only), and the signal from this well was used as the baseline for the other wells of the same plate. The instrument automatically decomposes the impedance signal into resistance and capacitance. As the course of capacitance at 64 kHz mirrored that of resistance at 2 kHz, the observed evolution of the resistance signal reflects changes in the cell-surface contact area. The ECIS records were exported to Microsoft Excel and processed using the GraphPad Prism software: the background was set to zero at a time point shortly before cell seeding, and the baseline (empty well) was subtracted. The curves shown in the graphs represent the averages from replicate wells, which were run in parallel.

Statistical analyses. As described in our previous work[36], the majority of experiments were performed using cell lines and repeated until the observed differences between groups reached statistical significance. A p value of 0.05 or lower was pre-set to be indicative of a statistically significant difference between groups compared. In diagrams, arithmetic means of replicates of all experiments were plotted with SD error bars. Significance levels (p values of ANOVA or Student's t-test) were determined using InStat Software (GraphPad Software).

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Author contributions

B.B., M.Š., P.H., A.H. and K.K. conceived the experiments; A.H. and M.Š. constructed plasmids and transfected cells; B.B. and M.Š. performed immunoprecipitation and western blot analysis; B.B., A.H. and M.Š. made confocal microscopy experiments and analysis; P.H., D.S. and A.H. measured and analyzed the FLIM-FRET data; P.O. performed native electrophoresis; K.K. performed and analyzed ECIS experiments; D.G. made and analyzed adhesion signaling-related blots; M.Š. and B.B. wrote the paper; B.B., P.H., K.K. and A.H. edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Dodatkové informace k publikaci č. 4: NSC348884 cytotoxicity is not mediated by inhibition of nucleophosmin oligomerization

NSC348884 cytotoxicity is not mediated by inhibition of nucleophosmin oligomerization

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Supplementary information 1: Supporting figures

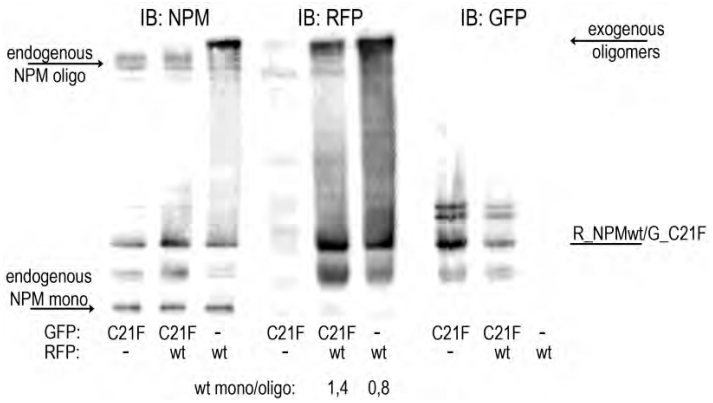


Fig. S1: Effect of C21F substitution on the stability of heterooligomers with NPMwt. Western blots of semi-native PAGE of samples from 293T cells transfected with R_NPMwt (wt), G_C21F (C21F) or with their combination. Similar results were obtained with the inverse labeling combination, i.e. with G_NPMwt and R_C21F.

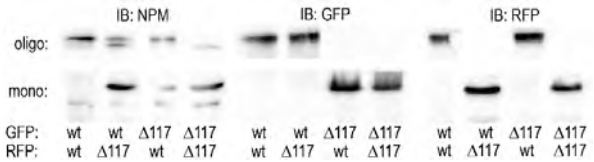


Fig. S2: Significance of the N-terminus for NPM oligomerization. Semi-native PAGE of 293T cells co-transfected with various combinations of Δ117 and NPMwt illustrates absence of interaction between Δ117 and NPMwt.

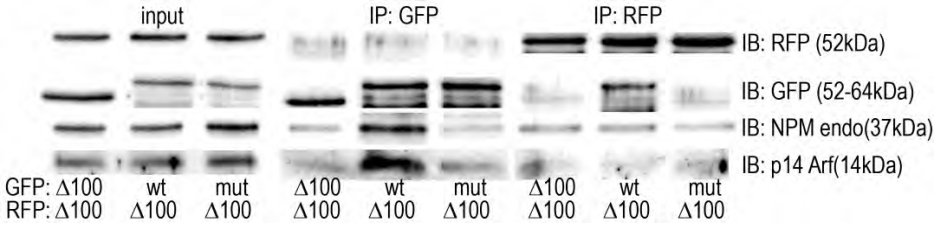


Fig. S3: Significance of the N-terminus for NPM oligomerization. Lysates from 293T cells co-expressing GFP-labeled Δ100/NPMwt/NPMmut and RFP-labeled Δ100 were subjected to GFP- and RFP-immunoprecipitation. The levels of co-precipitated interaction partners were investigated.

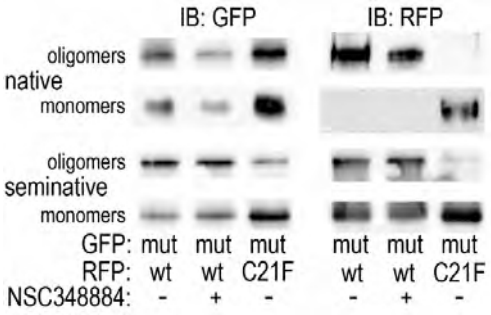


Fig. S4: Native (upper row) and semi-native (lower row) PAGE from 293T cells transfected with various fluorescent variants of NPM and treated with 10μM NSC348884 for 24h. Cells co-transfected with G_NPMmut and R_NPMwt (lanes 1 and 2) or R_C21F (lane 3). G_NPMmut and R_NPMwt/C21F are detected in the untreated (lanes 1 and 3) or NSC348884-treated cells (lane 2) by anti-GFP and anti-RFP antibodies.

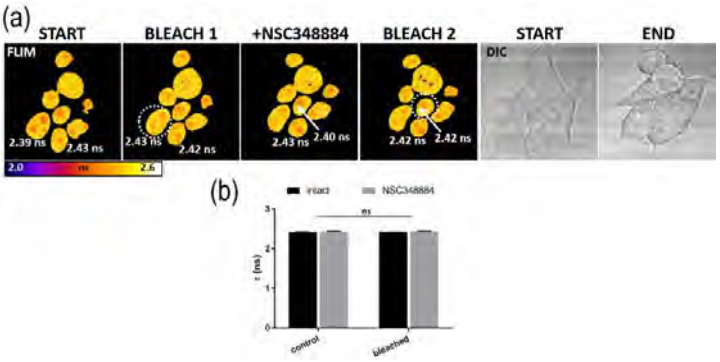


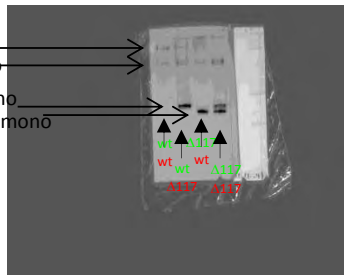
Fig. S5: NCL expression in live cells after NSC348884 treatment. (a) FLIM-FRET analysis of eGFP-fluorescence lifetime (τ) during 2h of 10μM NSC348884 action on cells co-transfected with donor- and acceptor-labeled variants of NCL. White numbers: τ (ns) measured in individual cells. Dashed circles: region of the acceptor (mRFP1) photobleaching. Simultaneous cell morphology screening by DIC documented cell rounding induced by NSC348884. (b) Statistical evaluation of τ before (control) and after (bleached) mRFP1-photobleaching in intact (black bars) and NSC348884-treated (grey bars) cells. Student´s t-test of the “control” vs. “bleached” values: not significant.

Supplementary information 2: full-length blots

Fig. 5:

(b):

NPM oligo&mono:



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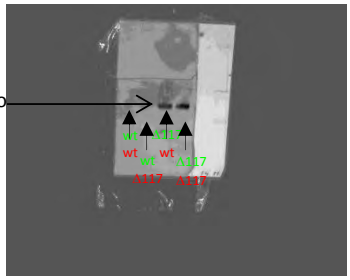
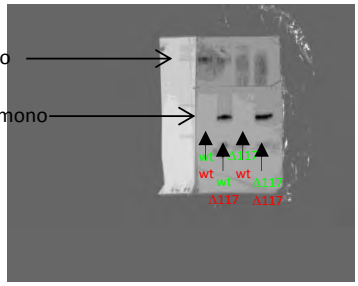


GFP oligo&RFP mono:



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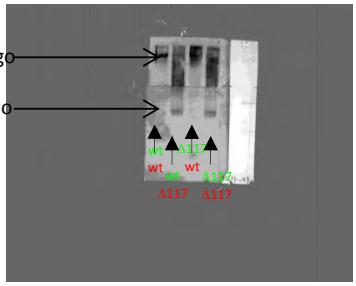
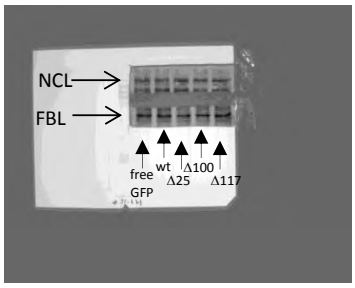


(c):

NCL&FBL Input:



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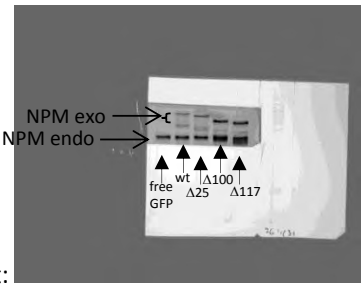

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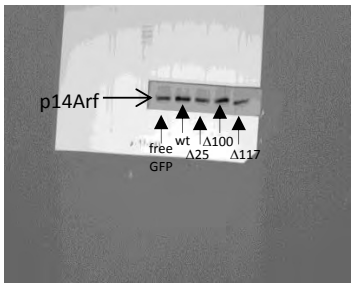

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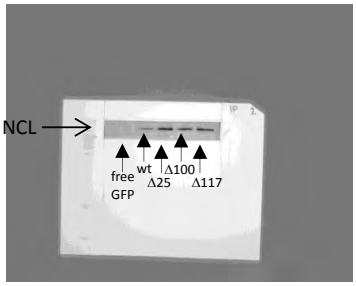

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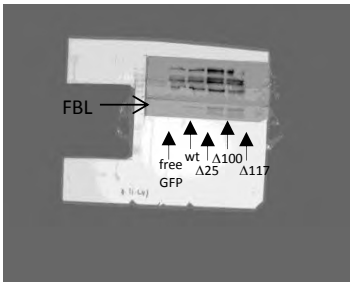

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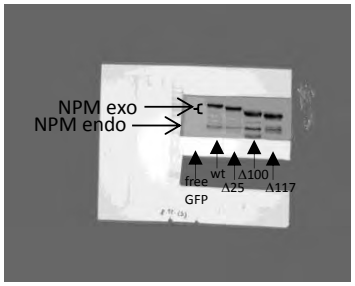

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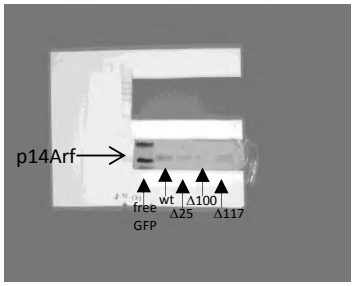
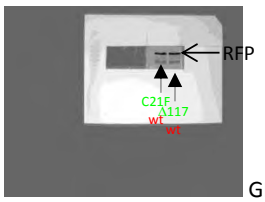


p14Arf IP (enhanced):

(d):

Input: RFP



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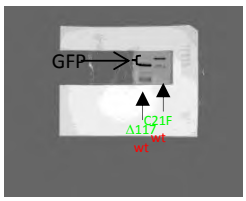
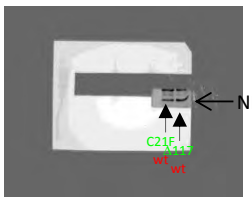
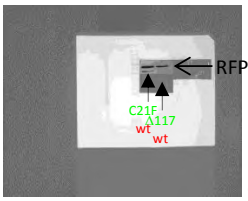


NPM



IP_GFP: RFP



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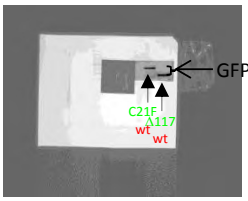
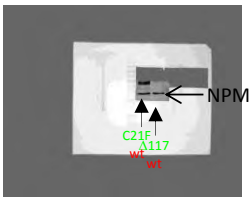
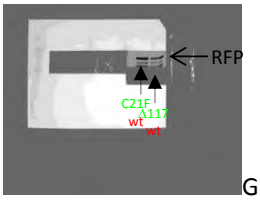


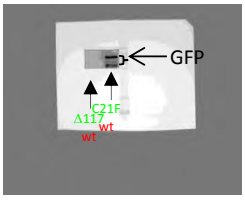
NPM



IP_RFP: RFP



GFP



NPM

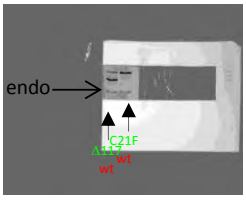
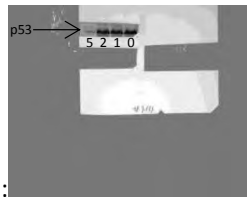
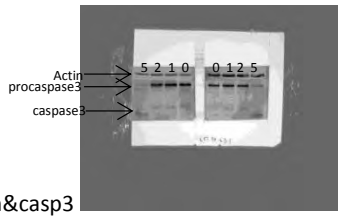
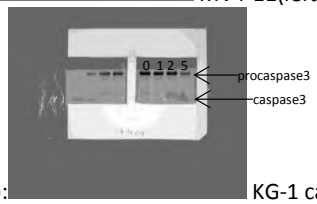


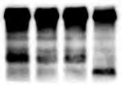
Fig. 7:

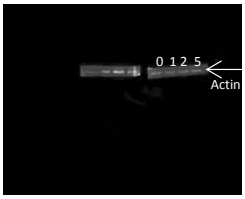
(b):

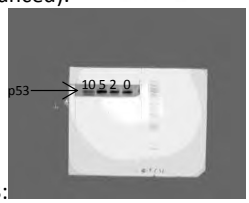
MV4-11 p53: 

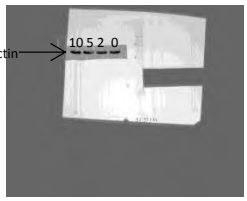
MV4-11(left)&HL-60(right) β-Actin&casp3 

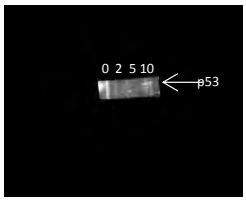
KG-1 casp3 (right): 

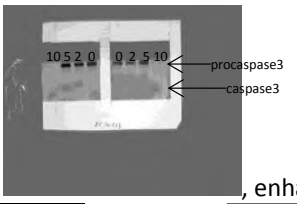
KG-1 casp3 (enhanced): 

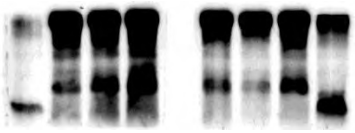
KG-1 β-Actin (right): 

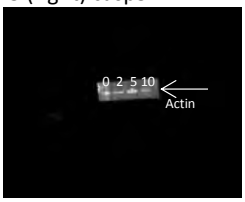
OCI-AML2 p53: 

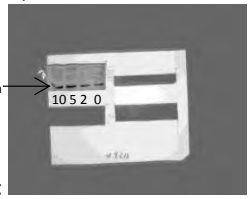
OCI-AML2 β-Actin: 

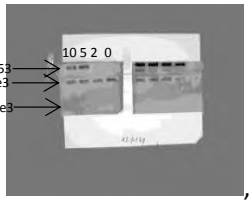
OCI-AML3 p53: 

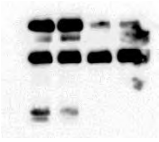
OCI-AML2 (left) and 3 (right) casp3: 

, enhanced: 

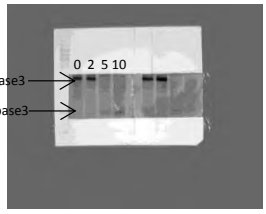
OCI-AML3 β-Actin: 

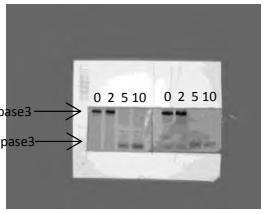
HeLa β-Actin: 

HeLa p53&casp3 (left): 

, enhanced: 

(c):

#41 (left) 

#42&43 

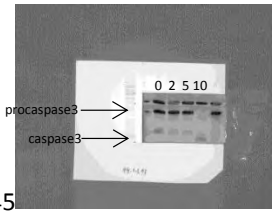
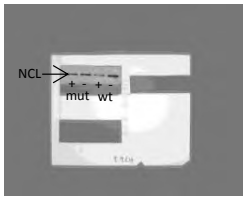
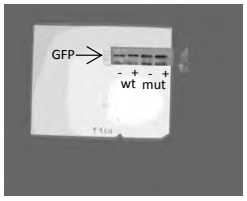
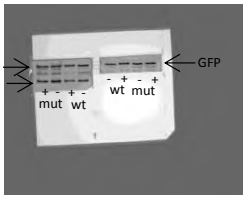
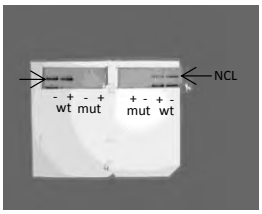
#45 

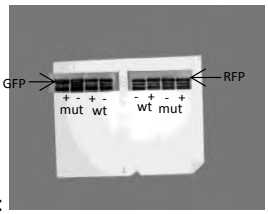
Fig. 11:

NCL (Input): 

GFP (Input): 

NPM&RFP (Input): 

NCL (IP_GFP&IP_RFP): 

GFP (IP_RFP)&RFP (IP_GFP): 

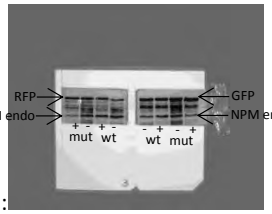
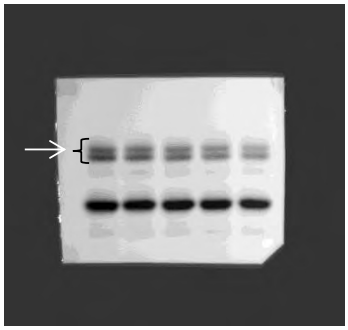
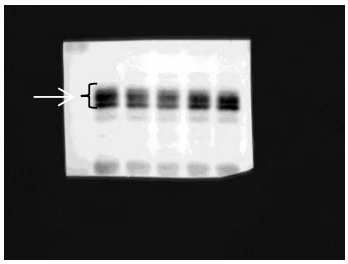
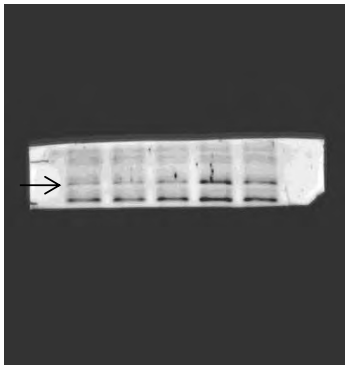
RFP&NPM (IP_RFP) & GFP&NPM (IP_GFP): 

Fig.14:

pPAK1 Ser14 

PAK1 

pCofilin Ser3 

Cofilin 

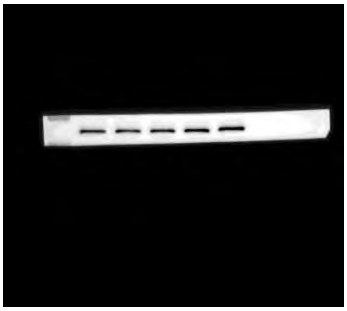
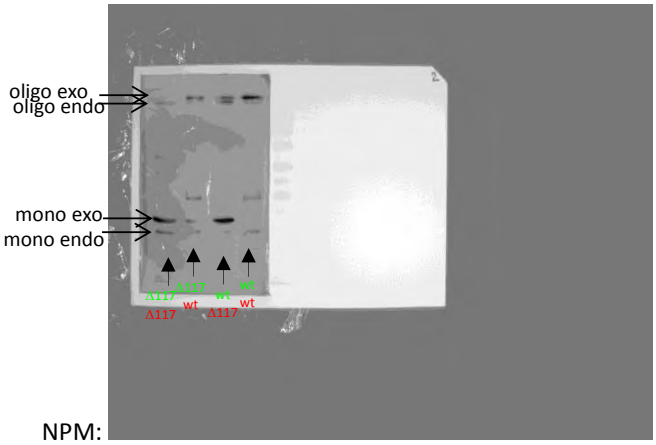
β-Actin 

Fig. S2

NPM:



GFP and RFP:

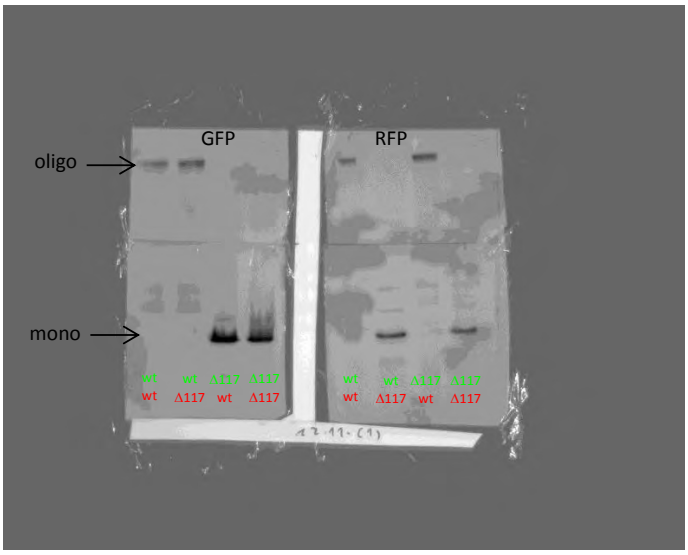
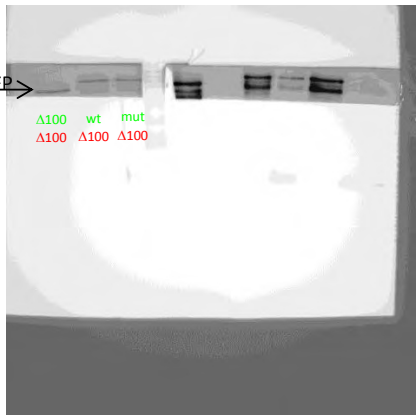
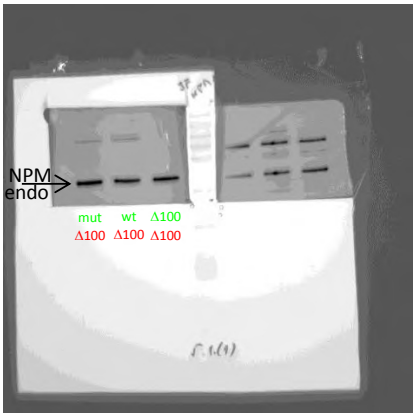
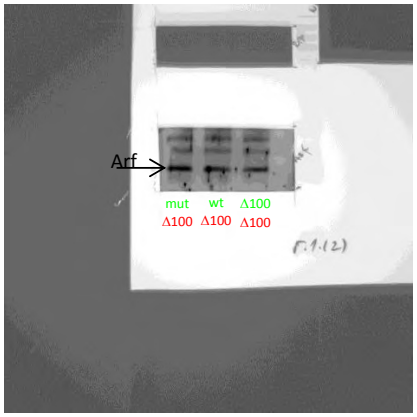


Fig. S3:

RFP input: 

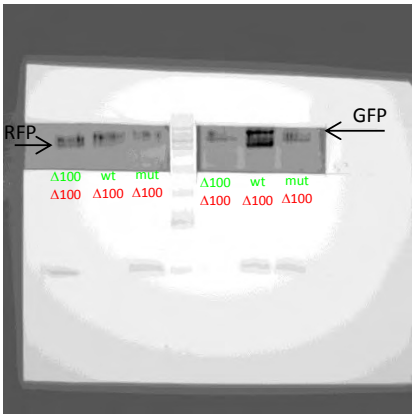
GFP input: 

NPM input: 

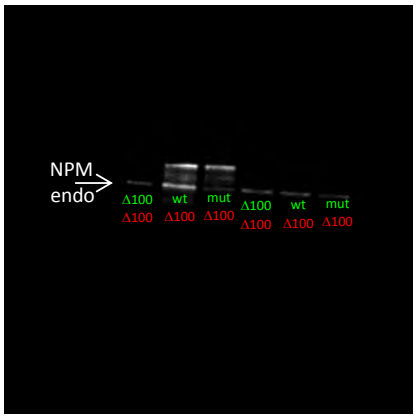
Arf input: 

GFP IPG & RFP IPR: 

RFP IPG & GFP IPR:



NPM IP:



Arf IP:

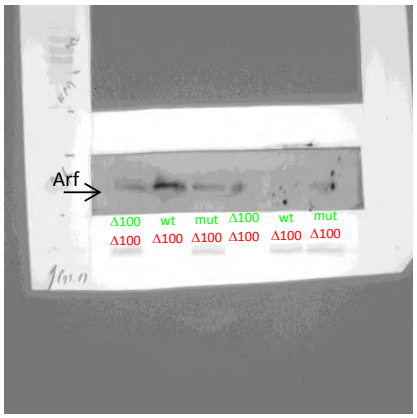
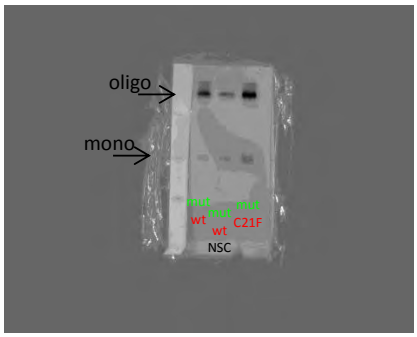
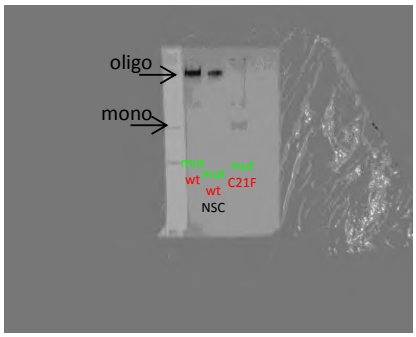


Fig S4:

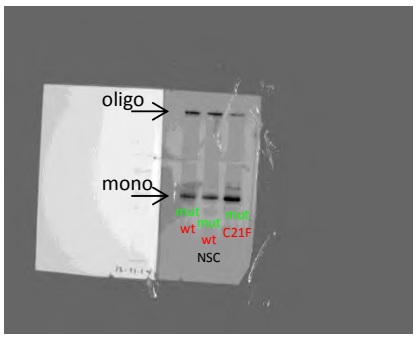
GFP native:



RFP native:



GFP seminative:



RFP seminative: