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Úloha transkripčního faktoru Snail v mechanismu rozvoje radiorezistence u nádorových linií karcinomu prostaty

Role of transcription factor Snail in mechanism of development of radioresistance in prostate carcinoma cell lines

Diplomová práce

Vedoucí závěrečné práce: MUDr. Zdeněk Hodný, CSc.

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Prohlášení

Prohlašuji, že jsem diplomovou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Abstract

The frequent cause of failure of prostate carcinoma radiotherapy and chemotherapy is the emergence of resistance and a progress into the essentially incurable metastatic form of disease. Although the mechanisms of the radioresistance and chemoresistance are still not well understood, recent studies indicate that transcription factor Snail, a key mediator of the epithelial-mesenchymal transition and subsequent metastasis formation, plays a critical role in the development of the chemoresistance and radioresistance in the tumor cells. As the activation of the optimal DNA damage response pathway is the determining factor for the cell survival after chemotherapy and radiotherapy, we hypothesized the role of Snail in the transcription regulation of these processes. In this study, we first analyzed the relationship between Snail and ATM kinase, as the ATM was recently reported to regulate stability of Snail by its phosphorylation. Although, we observed a modest effect of ATM inhibition on Snail levels after cancer cells exposure to ionizing radiation, we did not fully reproduced the recently published findings. Furthermore, we evaluated the role of Snail in transcription regulation of cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$. Our data point towards the suppressive role of Snail in $p21^{waf1/cip1}$ regulation, independent on the status of tumor suppressor p53. Finally, we attempted to identify the novel Snail transcriptional target genes, specifically those involved in the DNA damage response. Based on presence of putative Snail DNA binding elements (E-boxes) in their promoter regions, we selected two factors known to function in DNA damage response and cell cycle regulation - hSSB1 and CCNB3 - as potential transcription targets of Snail. However, manipulating Snail levels by ectopic overexpression or knock-down by RNA interference had no effect on mRNA levels of these two selected genes.

Keywords: prostate carcinoma, radioresistance, DNA damage response, Snail, epithelial-mesenchymal transition, radiotherapy

Abstrakt

Častou příčinou selhání léčby karcinomu prostaty je rezistence vůči radioterapii a chemoterapii s následným rozvojem metastatické, a v podstatě neléčitelné, formy onemocnění. Ačkoli mechanismy rozvoje radiorezistence nebyly doposud zcela objasněny, některé studie ukazují, že transkripční faktor Snail, klíčový mediátor epiteliálně-mezenchymální tranzice i následné tvorby metastáz, má zásadní roli v rozvoji chemorezistence a radiorezistence nádorových buněk. Protože aktivace optimální odpovědi na poškození DNA je určujícím faktorem pro přežití buněk vystavených chemoterapii či ionizujícímu záření, předpokládali jsme roli Snail právě v těchto procesech. V této práci jsme se nejdříve zabývali analýzou vztahu mezi Snail a kinázou ATM. Přestože nedávné studie naznačují, že ATM může regulovat stabilitu Snail skrze jeho fosforylaci, a taktéž my jsme pozorovali mírný vliv inhibice ATM na hladiny Snail u nádorových buněk vystavených ionizujícímu záření, na základě souhrnných výsledků ze všech námi provedených experimentů nelze tuto regulaci jednoznačně potvrdit. V dalším kroku jsme hodnotili roli Snail v regulaci transkripce cyklin-dependentní kinázy $p21^{waf1/cip1}$. Výsledná data poukazují na to, že v regulaci transkripce $p21^{waf1/cip1}$ má Snail supresivní účinek, kterýžto není závislý na funkčním stavu nádorového supresoru p53. Nakonec jsme se pokusili identifikovat nové transkripční cíle Snail, které by se zároveň podílely na regulaci odpovědi na poškození DNA. Na základě přítomnosti DNA-vazebných sekvencí pro Snail (E-boxů) na promotorech vybraných genů jsme určilili dva faktory účastnící se odpovědi na poškození DNA a regulace buněčného cyklu, hSSB1 a CCNB3, jako potenciální transkripční cíle Snail. Avšak, manipulace s hladinou Snail, zvýšením či snížením jeho exprese, neovlivnila hladinu mRNA těchto dvou vybraných genů.

Klíčová slova: karcinom prostaty, radiorezistence, odpověď na poškození DNA, Snail, epiteliálně-mesenchymální tranzice, radiotherapie

Content

List of Abbreviations

AD	Androgen-dependent
AI	Androgen-independent
AR	Androgen receptor
AS	Androgen-sensitive
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
BCA	Bicinchoninic acid
BrdU	5-Bromo-2´-deoxyuridine
CCNB3	Cyclin B3
Cdk(s)	Cyclin-dependent kinase(s)
ChIP-on-chip	High-throughput chromatin immunoprecipitation and promoter array analysis
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
Chk2pT68	Chk2 kinase phosphorylated on threonine 68
CPT	Camptothecin
DAPI	4',6-diamidino-2-phenylindole staining
DDR	DNA damage response
DSB(s)	Double-strand break(s)
DMEM	Dulbecco's modified Eagle's medium
dox	Doxycyclin
E-cadherin	Epithelial cadherin
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR2	Fibroblast-growth-factor receptor-2
FSP1	Fibroblast-specific protein-1
fIR	Fractioned ionizing radiation
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GSK-3β	Glycogen synthase kinase-3β
Gy	Gray
HDRB	High-dose rate brachytherapy
HGF	Hepatocyte growth factor
HR	Homologous recombination
IR	Ionizing radiation
LDRB	Low-dose rate brachytherapy
LFC	Log fold change
MET	Mesenchymal-epithelial transition
MF(s)	Microfilament(s)
MMEJ	Microhomology-mediated end joining
MMP(s)	Matrix metalloproteinase(s)
Mre11	Meiotic recombination 11 homologue
MRN	Mre11-Rad50-Nbs1
Nbs1	Nijmegen breakage syndrome 1
NCS	Neocarzinostatin
NES	Nuclear export sequence
NHEJ	Non-homologous end joining
p16	$p16^{INK4a}$
p21	$p21^{waf1/cip1}$
$p53^{-/-}$	p53 null
$p53^{wt/wt}$	p53 wild-type
Pak1	p21-activated kinase 1
PBS	Phosphate buffered saline
PCa	Prostate cancer
PCR	Polymerase chain reaction
PLND	Pelvic lymphadenectomy
PSA	Prostate-specific antigen
RB	Retinoblastoma protein
qRT-PCR	Quantitative real time reverse transcription polymerase chain reaction
ROS	Reactive oxygen species
RP	Radical prostatectomy
RPMI	Roswell Park Memorial Institute

RT	Room temperature
SA-β-al	Senescence-associated β-galactosidase
SASP	Senescence-associated secretory phenotype
SD	Standard deviation
siNC	Non-targeting siRNA
siSN, siSnail	Snail siRNA
SRD	Serine-rich domain
SSB(s)	Single-strand break(s)
SSBP1, (h)SSB1	(human) Single strand binding protein 1
TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor α
UV	Ultraviolet
VEGF-A	Vascular endothelial growth factor-A
ZF	Zinc-finger

1. Introduction

The major cause of cancer-related mortality is development of metastasis (Gupta and Massagué, 2006). In tumors of epithelial origin - carcinomas, the initial step of the invasion-metastasis cascade includes activation of the epithelial-mesenchymal transition (EMT). One of the main mediators of EMT is a zing-finger family transcription factor Snail (Moody et al., 2005). It was shown that Snail binds to a specific consensus sequence (5´-CANNTG-3´) in the promoter of the epithelial cell-to-cell contact factor E-cadherin and works as a repressor of its transcription (Cano et al., 2000). E-cadherin repression is an important step in the tumor progression since its downregulation leads to the acquisition of mesenchymal properties including increased motility and invasiveness, resulting in cancer cell dissemination to the distant organs of the body (Frixen et al., 1991). Moreover, Snail was shown to be overexpressed in the variety types of carcinomas (Blanco et al., 2002; Rosivatz et al., 2002; Sugimachi et al., 2003), especially in patients undergoing anti-cancer chemotherapy and/or radiotherapy and its increased expression has been associated with the worse prognosis (Muenst et al., 2013; Shin et al., 2012). The role of Snail in EMT as well as its role in acquired chemoresistance and radioresistance is well-studied (Hoshino et al., 2009; Kurrey et al., 2009; Kyjacova et al., 2015), however, the functional link between Snail and DNA damage response (DDR) is not known in detail.

DDR represents a complex regulatory mechanism that enables cells to execute biological responses to DNA damage caused by various genotoxic insults. Besides DNA repair pathways, the DDR is believed to serve as the biological barrier that prevents early stages of tumorigenesis (Kastan, 2008; Nuciforo et al., 2007). Moreover, several studies suggest the role of factors involved primarily in DDR signaling in the development of chemoresistance and radioresistance in various tumor types (reviewed by Kastan and Bartek, 2004).

These findings indicate the mutual link among DDR and EMT, however, the molecular mechanisms of this linkage are still obscure. Our goal was to decipher the mutual relationship between the activation of DDR machinery by genotoxic insults (IR) and Snail expression as well as to specify the role of Snail in the regulation of DDR-induced cell cycle arrest and the expression of DDR effectors and cell cycle regulators involved in DDR.

2. Overview of literature

2.1. Prostate cancer

2.1.1. Prostate physiology

Prostate gland is one of the accessory glands of the male reproductive system. It is unpaired, located below the bladder and surrounds the urethra (Figure 1). Healthy prostate of adult male is small, about a size of a walnut. Its main role is to produce secretions that are mixed with sperm during ejaculation and that increase the chance of fertilization and sperm survival.

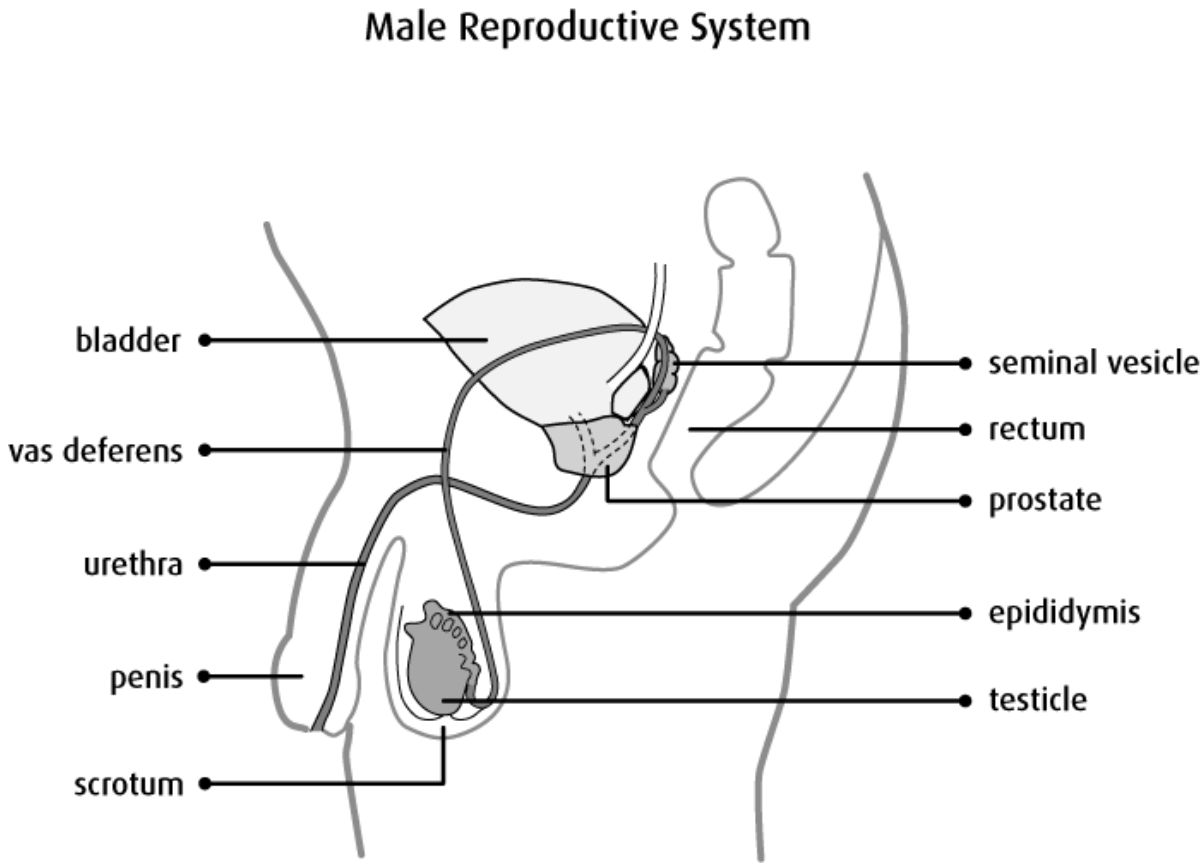
The product of the prostate, which makes up about a half of the seminal fluid volume, is thin and milky, contains various glycoproteins, alkaline compounds and small molecules such as prostaglandins and polyamines. Moreover, the fluid is rich in hydrolytic enzymes, notably fibrinolysin. The sugars secreted by the prostate serve as the nutrition for the sperms as they pass into the female body to fertilize the ovum. Enzymes break down proteins in the semen after the ejaculation in order to free sperm cells from the viscous semen. The alkaline chemicals in prostatic secretions neutralize the acidic vaginal secretions for the purpose of the enhancement of the survival of sperms in the female body (Mescher and Junqueira, 2013).

The prostate consists of the two main types of tissue. First of them is epithelial exocrine glandular tissue that makes up most of the prostate, as it is specialized for the secretion of the components of the semen. The other one is the fibromuscular tissue, composed of the mixture of the smooth muscle tissue and the dense irregular connective tissue. Its function is to provide the strength to the tissue and to expel the fluids (Mescher and Junqueira, 2013).

Prostate gland can be divided into 3 zones – peripheral, central and transition zone. The peripheral zone is the area of the prostate that is notably in the dorsal part closest to the rectum. It is the largest zone of the prostate gland and the majority of prostate tumors (approximately 75%) are found in this zone – that is why the prostate cancer (PCa) is accessible for the rectal examination – *per rectum*. The transition zone is located in the middle of the prostate, between the peripheral and central zones. It surrounds the urethra as it passes through the prostate. This zone makes up about 20% of the prostate gland until the age of 40. As men reach the ripe age, the transition zone gradually enlarges - until it becomes the largest area of the prostate. This is called benign prostatic hyperplasia. When the transition zone enlarges, it pushes the peripheral zone of the prostate towards the rectum, and this phenomenon can be easily felt during the rectal

examination. Central zone is in the front of the transition zone. It is the part of the prostate that is the farthest from the rectum (McNeal, 1981).

A clinically important product of the prostate is the prostate-specific antigen (PSA), a 34-kDa serine protease, whose function is to liquefy the coagulated semen. Therefore, the highest concentration of PSA can be found in the semen and only a small amount of it is released into the bloodstream. But if the structure of cells or glandules is damaged, PSA may be released into the blood at a higher amounts. The occurrence of this can point to pathological processes in the prostate, such as the inflammation or even cancer. Because of its easy detection in the blood and its relatively high significance, the PSA testing was introduced as a screening tool for early prostate cancer detection into the clinical practice since the 1994 (Catalona et al., 1994).



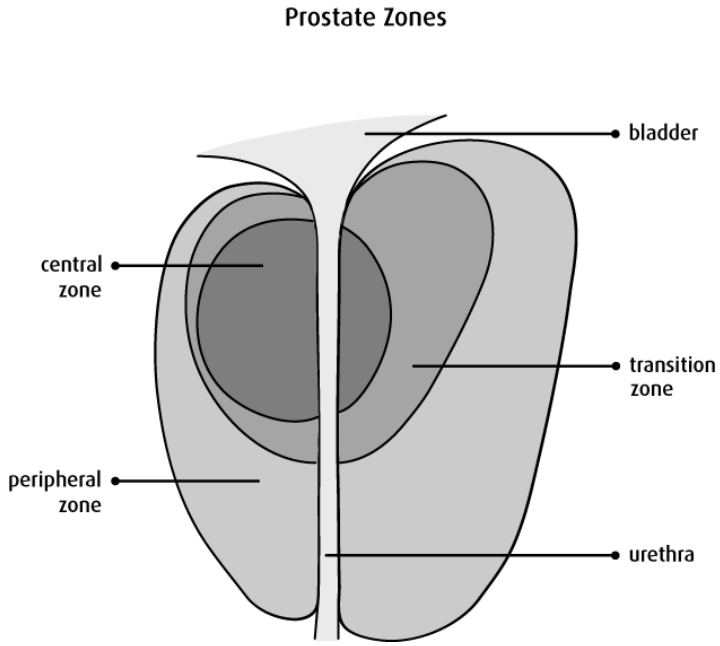


Figure 1. The image above shows the prostate gland and the nearby organs in the male reproductive tract. The image below shows 3 prostate zones and depicts how the prostate surrounds the urethra. (http://www.cancer.ca/en)

2.1.2. Prostate cancer

The term „cancer“ means a condition, when normal cells lose their ability to regulate cell growth and they are not able to undergo a cell death. It leads to the accumulation of extra cells that often form a mass of tissue called tumor. Tumors are divided into two categories, benign and malignant. Benign tumors are usually not life-threatening, since they do not invade a tissue around them and cannot expand to the other parts of the body. These tumors can be removed from the body without the chance of their re-growth. Contrary to this, malignant tumors can spread to the other parts of the body and invade nearby tissues and organs. These tumors are often life-threatening and it is not so easy to remove them permanently and completely, because they are usually not well demarcated and will regrow back in many cases.

PCa is one of the most prevalent malignancy in males (Plata Bello and Concepcion Masip, 2014) with the incidence higher than that of all the other solid tumors (Figure 2). Every year, nearly 1 million of new cases is being diagnosed worldwide, of which a one-third are fatal (Ferlay et al., 2010a). The incidence rates of PCa fluctuate worldwide, depending on the geographic region and race, which is partly due to the prevalence of PSA testing and with the digital rectal examination performance in men of a certain age groups. The highest rates of new cases are estimated in the Western countries, such as in the USA (namely Afroamerican population), in the Northern and Western European countries (Ferlay et al., 2010b) and in Australia/New Zealand. In comparison with the incidence, the mortality rates vary much less. According to the data available for 2007 in the Czech Republic, the estimated incidence is 100.2/100.000 and the mortality of 25.1/100.000 (Dusek et al., 2010).

Nowadays, more than 70% of PCa cases are diagnosed by the occurrence of the elevated PSA levels and approximately 10 years earlier than before the PSA was introduced into the standard clinical practice (Schröder et al., 2012). The widespread implementation of PSA testing allows for the early start of the effective treatment. These two factors contribute to the relatively low rate of the mortality, when compared to the incidence of this type of cancer.

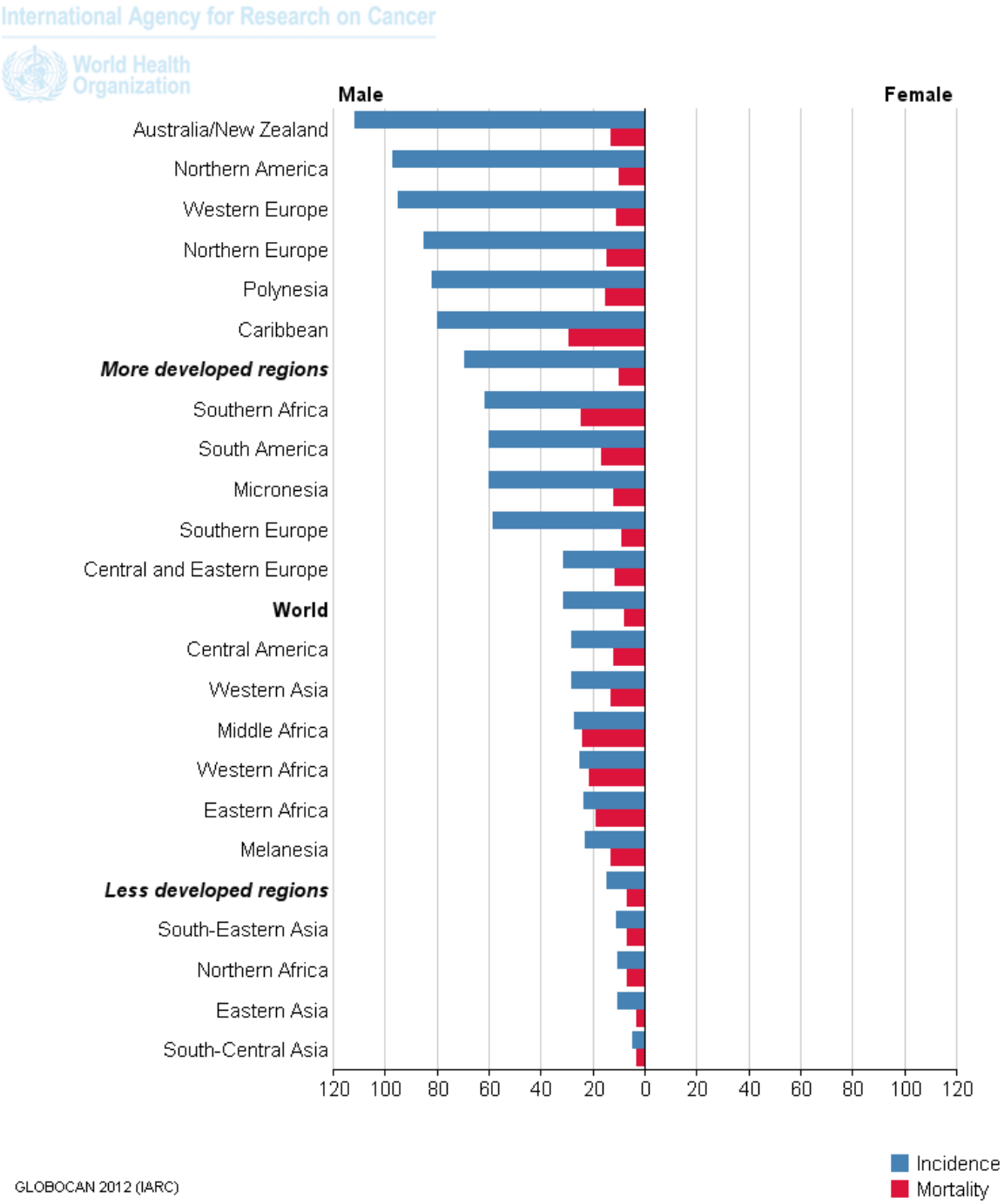


Figure 2. Worldwide prostate cancer incidence and mortality. The estimated age-standardized rates (world) per 100.000. Data are available from Globocan2012. While the incidence rates are much higher in developed countries, the associated mortality rates are relatively low. However, in developing countries, even though the incidence rates appear to be low, the associated mortality rates are almost similar to the incidence rates, indicating a high rate of fatality due to the prostate cancer in these countries (http://www.globocan.iarc.fr/Pages/fact_sheets_cancer.aspx).

PCa incidence increases with age. It is predominant in men above 40, with about 60% of cases diagnosed in men older than 65 years (Figure 3). The growth of early PCa is supported by 5α-dihydrotestosterone (5α-DHT) - the derivate of an androgen testosterone, produced by the Leydig cells in the testis. Thus, this type of cancer may arise in each man with the active production of sex hormones. The epidemiological studies done in the populations who had migrated to another continent suggest that the lifestyle and the environment also determine the prostate cancer risk (Lee et al., 2007). Risk factors include the alcohol consumption, smoking, eating animal fat, obesity, low physical activity, drug medication, low vitamin intake (e.g. vitamin D and E) and high mineral intake (zinc, calcium, selenium), and finally low sexual activity (Giovannucci et al., 2007; Hebert et al., 1998; Huncharek et al., 2010; Lawson et al., 2007; Rota et al., 2012; Schwartz, 2013).

Another major risk factor for PCa is a genetic predisposition. This term covers family history and race. Some studies have shown that the preponderance of PCa incidence as well as the mortality from PCa is much higher among black men than in Caucasian men (Virnig et al., 2009). Moreover, it has been observed that PCa has a high probability of heritability (Alberti, 2010). For example, the first degree relatives of PCa patients were at double the risk of developing PCa as the normal population (Goldgar et al., 1994). Several genes have been identified as they increase the likelihood of PCa incidence (Simard et al., 2003).

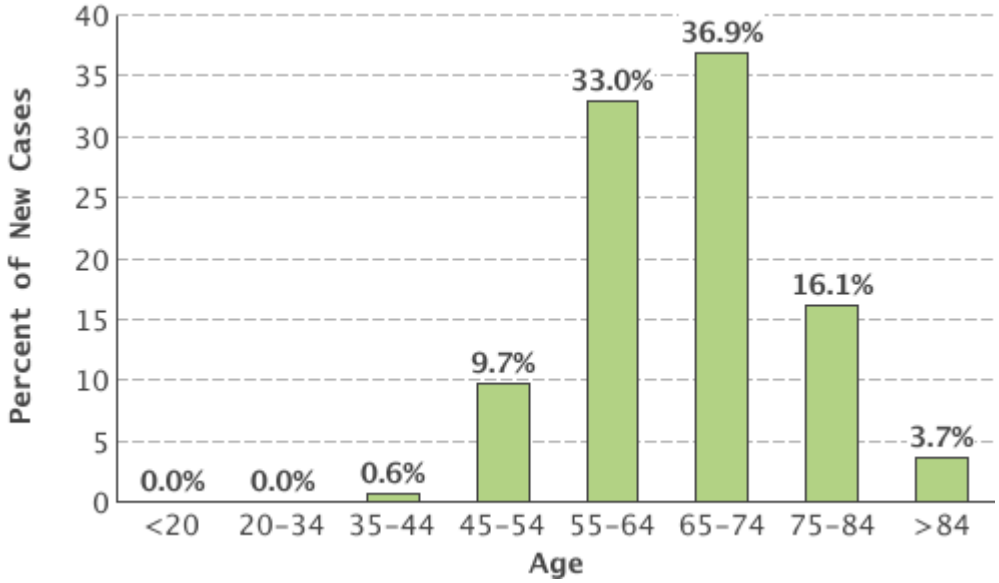


Figure 3. Diagnosis of new cases of prostate cancer. Based on data between years 2008 – 2012, US population. All races are included. The prostate cancer is most frequently diagnosed among men aged 65-74. The median age of the diagnosis is 66 years (http://www.seer.cancer.gov/statfacts/).

2.1.3. Prostate cancer treatment strategies

PCa is mostly asymptomatic tumor with a slow growth rate. A very low mortality rate relative to the incidence of PCa appears to be due to a high rate of over-diagnosis and overtreatment. The disease progression varies among individuals, allowing for stratified approaches towards the definitive treatment (reviewed by (Chen and Zhao, 2013)). Although about a two-third of PCas are slow-growing, there are still present some cases with the aggressive phenotype. The choice of treatment depends largely on several factors, like the stage of the progression, the initial PSA level, patient's age and co-morbidities.

In case of older asymptomatic men or in patients with other medical conditions, the most useful treatment is watchful waiting and the active surveillance. Both treatment options include closely monitoring a patient's condition without giving any treatment unless there are changes in test results, or until signs or symptoms appear or change.

Surgery is suggested primarily for high-risk, localized PCa (Lawrentschuk et al., 2010) and it is applied as a part of the multimodality approaches rather than as a monotherapy. The most commonly performed types of the surgery in PCa are: radical prostatectomy (RP), which include removal of the prostate together with surrounding tissue and seminal vesicles and pelvic lymphadenectomy (PLND), where lymph nodes in the pelvis are removed to detect potential lymph node metastases. PLDN is usually performed during RP for high risk prostate cancer (Koupparis and Gleave, 2010). Surgery has been shown to reduce the risk of death, metastases and local tumor progression in 10-year period (Bill-Axelson et al., 2005). However, there are some side effects that discourage this type of PCa treatment. Possible problems after PCa surgery include impotence, leakage of urine from the bladder or stool from the rectum, inguinal hernia or penis shortening (Ratcliff et al., 2013).

After RP, radiation therapy is considered as the second major therapeutic modality for high-risk locally advanced PCa. The widely used radiotherapy strategies for PCa are external radiotherapy and brachytherapy. External radiotherapy uses a machine outside the body to apply radiation toward the tumor. The gradual increase in the dose of radiation, up to 78 Gy with daily fractions of 2 Grays (Gy) represent standard protocol for men with localized PCa (Kuban et al., 2008). External radiotherapy is effective to patients without distant metastases. For patients with local but more advanced disease, brachytherapy has been shown to be a better treatment strategy (Law and McLaren, 2010). Low-dose rate brachytherapy (LDRB) involves the permanent placement of the radioactive seeds guided by ultrasound with half-life of 60 days into the patient's body near the tumor. In comparison, high-dose rate brachytherapy (HDRB)

means a temporary insertion of applicators into the prostate. This ensures a high dose of radiation to prostate gland, but the minimized dose to nearby organs (Law and McLaren, 2010). The negative side effect of radiotherapy is erectile dysfunction, which may develop progressively in the eldery population (Karlsdóttir et al., 2008). Moreover, radiation treatment increases the risk of bladder and/or gastrointestinal cancer (Stokkevåg et al., 2015).

Another common treatment option for PCa is hormone therapy that is initiated by reducing the concentration of circulation androgens. Testosterone, which is produced mainly in the testes, and its more potent metabolite dihydrotestosterone are androgens that bind and activate the androgen receptor - androgen-activated transcription factor and a member of the steroid receptor subfamily of the nuclear receptors (Freeman et al., 2001). Hormone therapy targets the androgen receptor, because androgen-androgen receptor signaling is required for growth and survival of hormone-sensitive prostate cancer cells (see below). Hormonal therapy reduces the androgen concentration through surgical or medical castration or by administering anti-androgens (Labrie et al., 1982). Among side effects of hormonal therapy belongs impaired sexual function, loss of desire for sex, weakened bones, etc (Ahmadi and Daneshmand, 2013).

Chemotherapy as a cancer treatment that uses drugs to stop the growth of cancer cells is not considered as a very effective way to battle PCa. Nevertheless, chemotherapy is used for patients with hormone refractory PCa, as it brings improvements in pain and quality of life as well as decreases in PSA level (Picard et al., 2012).

Although cancer treatment has important impacts on quality of life (White et al., 2013), the overall quality of life for PCa patients has improved from 1999 to 2011 (Glass et al., 2013). Current treatment options for PCa include surgery in combination with hormonal and radiation therapies, nevertheless, the optimal treatment choice is based on many factors, including initial PSA level and clinical stage of disease, together with baseline urinary function, comorbidities, and patient age.

2.1.4. Prostate cancer cell lines as experimental model

Prostate cancer is very heterogeneous disease and its biological, molecular and hormonal characteristics are immensely complex. This, together with tendency of PCa cells to metastasize to various organs, provides the basis for the introduction of many different PCa cell lines as experimental models of PCa. Table 1. contain the list of the major human prostate cancer and immortalized cell lines.

Cell line	**Source**
PC-93	AD primary prostate cancer
PC-3	Lumbar metastasis
DU-145	Central nervous system metastasis
TSU-Pr1*c*	Cervical lymph node metastasis in Japanese male
LNCaP	Lymph node metastasis in Caucasian male
LNCaP-FGC*d*	Clonal derivative of LNCaP
LNCaP-LN-3	Metastatic subline of LNCaP cells derived by orthotopic implantation
LNCaP-C4	Metastatic subline of LNCaP derived after coinoculation of LNCaP and fibroblasts
LNCaP-C4B	Metastatic subline derived from LNCaP-C4 after reinoculation into castrated mice
MDA PCa 2a	AI bone metastasis from African-American male
MDA PCa 2b	AI bone metastasis from African-American male
ALVA-101	Bone metastasis
ALVA-31*e*	Well-differentiated adenocarcinoma
ALVA-41*e*	Bone metastasis
22Rv1	Derived from CWR22R an androgen-dependent prostate cancer xenograft line
ARCaP	Derived from ascitic fluid from a patient with metastatic disease
PPC-1*e*	Poorly differentiated adenocarcinoma
LAPC3	Derived from xenograft established from specimen obtained via transurethral resection of the prostate
LAPC4	Derived from xenograft established from a lymph node metastasis
P69SV40T	Immortalized cell line derived by transfection of adult prostate epithelial cells with the SV40 large T antigen gene
RWPE-2	Immortalized cell line initially derived by transfection of adult (Caucasian) prostatic epithelial cells with human papillomavirus 18, then made tumorigenic by infection with v-K-ras
CA-HPV-10	Immortalized cell line derived by human papilloma virus 18 transfection of prostatic epithelia cells from a high-grade adenocarcinoma
PZ-HPV-7	Immortalized cell line derived by human papilloma virus 18 transfection of normal prostatic peripheral zone epithelial cells

Table 1. List of established human prostate cancer and normal immortalized cell lines (taken from (Russell and Kingsley, 2003)).

As mentioned above, 5α-DHT, the derivate of androgen testosterone, is required for development of PCa. Prostatic cells whose growth requires the presence of 5α-DHT are termed androgen dependent (AD). Androgen-sensitive (AS) are those PCa cells, which do not require androgens for their growth, but they can respond to it. The necessity of androgen presence is the reason for using hormonal therapy, especially for patients whose tumors are not operable (Rambeaud, 1999). Unfortunately, after period of remission the disease regresses almost invariably. Moreover, these new tumor cells become often androgen-independent (AI), which may be caused by changes in the androgen receptor, mostly by mutation (Wang and Uchida, 1997).

In this thesis, 3 types of prostate cancer cell lines were used – DU145, PC-3 and LNCaP. These lines have been chosen because of their different status in p53 gene expression (see below).

DU145 cell line has been derived from a human PCa metastasis to the brain. DU145, as well as PC-3, are AI (although express AR mRNA) (Alimirah et al., 2006) and do not express PSA and testosterone-5-α-reductase enzyme, which is responsible for production of 5α-DHT from testosterone. DU145 cells also synthesize detectable amounts of p53 protein that is, however, mutated in DNA binding domain and has an extended half-life (Isaacs et al., 1991). In addition, this line shows mutation in gene coding for inhibitor of cyclin dependent kinases, $p16^{INK4a}$ (p16) (Gaddipati et al., 1997). Karyotypic analysis has shown these cells to be aneuploid with a chromosome number of 64. DU145 cells have a moderate tumorigenic potential and exhibit characteristics of poorly-differentiated adenocarcinoma (Stone et al., 1978).

PC-3 cell line has been established in 1979 from bone metastasis of PCa in 62 year-old Caucasian male. These cells do not express PSA and lack testosterone-5-α-reductase and androgen receptors, which means that they are AI (Kaighn et al., 1979). PC-3 has been shown to contain mutation in p53 gene, namely they exhibit only one allele of chromosome 17p where is p53 located, which cause PC-3 appear to be p53 negative (using western blotting analysis) (Isaacs et al., 1991). Karyotypic analysis demonstrates an aneuploid human karyotype, comprising of 62 chromosomes. Further studies also revealed many features common to neoplastic cells of epithelial origin including numerous microvilli, junctional complexes, annulate lamellae and abnormal nuclei, nucleoli and mitochondria. PC-3 human PCa cell lines have high tumorigenic potential and produce poorly differentiated adenocarcinoma if inoculated into nude mice (Kaighn et al., 1979).

LNCaP cell line was derived in 1977 from left supraclavicular lymph node metastasis of PCa in 50-year-old Caucasian male. For a long time LNCaP cells were the only human PCa cell line that demonstrates androgen sensitivity. Cells also express PSA. LNCaP cells are aneuploid and they have a full complement of human chromosomes (Horoszewicz et al., 1980). Status of p53 in these cells is normal (non-mutated). They have a low metastatic potential and several laboratories observed LNCaP cells to be poorly tumorigenic in athymic mice unless coinoculated with tissue-specific stromal or mesenchymal cells (Gleave et al., 1992).

2.2. DNA damage signaling

2.2.1. DNA damage

Mechanisms that contribute to the preservation and transmission of genetic information across generations became the main subject of research since the discovery of DNA structure more than 60 years ago. It is clear that the protection of the DNA against damage is essential for the survival of all life forms on the Earth. Genetic information encoded by DNA is permanently exposed to various genotoxic insults, as well as the DNA structure is often disturbed during normal physiological processes. It, has been estimated that every cell could experience up to 10^5 spontaneous DNA lesions per day (Hoeijmakers, 2009). This included, *inter alia*, DNA breaks caused by reactive oxygen species (ROS) produced during normal cellular metabolism. Among exogenous sources of DNA damage belong physical and chemical agents. Ultraviolet (UV) light from sunlight, which can also induce up to 10^5 lesions per cell per day, and ionizing radiation (IR), generally used in cancer therapy, are examples of physical genotoxic insults (Hoeijmakers, 2009). The best known sources of chemical damage are drugs used in chemotherapy that can cause a huge spectrum of DNA lesion (alkylation, DNA-DNA and DNA-protein cross-links, single and double-strand breaks, etc.) The most common exogenous source of chemically induced DNA damage is cigarette smoking that causes an oxidative damage in lung and other tissues (Asami et al., 1997).

Among the variety of types of DNA damage, the most deleterious are the DNA double-strand breaks (DSBs). Permanent DSBs develop when the two complementary strands of DNA double-helix are disrupted to such an extent that does not allow their further association and subsequent reparation. The newly arisen DNA ends, if not adequately repaired by non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ) or homologous recombination (HR) are prone to invade other sites in the genome for incorrect recombination. In this case, DSBs can represent cause of cell death. If inaccurately repaired, DSBs may act as potential source of mutations that can support cell survival and even cancer development (Hsu et al., 2007; Moshous et al., 2003). DSBs are generated mainly by IR, chemotherapeutic agents or endogenously by the ROS (Zhang et al., 2009). However, in some cases, DSBs are generated and retroactively removed in a programmed manner during V(D)J recombination (Hendrickson et al., 1991), when the variety of antigen-binding receptors of

lymphocytes is created, or during meiosis, when parts of homologous chromosomes are mutually exchanged.

2.2.2. DNA damage response and repair

All living organisms are permanently exposed to DNA-damaging agents that induce various types of DNA damage. This includes base modification, DNA intrastrand, interstrand or DNA-protein crosslinks, single-strand breaks (SSBs) and DSBs. As cells need to adequately respond to each type of the genotoxic stress and to recognize all kinds of DNA damage, they have evolved mechanisms, which we collectively call DNA damage response (DDR). The main role of the DDR pathways is to stop cell-cycle progression and stimulate proper DNA repair of the damaged genome.

The cell cycle of proliferating cells is based on the repeating alternation of G1 (growth phase), S (DNA replication) and G2 (preparation for mitosis) phases, followed by mitosis (division of the nucleus) and cytokinesis (cellular division). Transition among the different phases of the cell cycle is managed by cyclin-dependent kinases (Cdk) and their interaction with various cyclins – such as Cdk2/cyclin-E complex in G1/S or Cdk1/cyclin-B complex in G2/M transition (Cerqueira et al., 2009). However, in case when the cell detects some defect through the cell cycle progression, the entering into the next phase is not allowed - Cdks are not activated or they are inhibited by Cdk-inhibitors and the cell cycle is arrested. This process is called as a checkpoint control and it is one of the most important parts of the DDR (Cerqueira et al., 2009).

The fast checkpoint induction after DNA damage is provided by a transmission of the signal through phosphorylation of multiple substrates by Ataxia telangiectasia Rad3-related/Ataxia telangiectasia mutated (ATR/ATM) and Checkpoint kinase 1/Checkpoint kinase 2 (Chk1/Chk2) kinases affecting the protein stability and/or activity of their target substrates. First Mre11-Rad50-Nbs1 (MRN) complex detects and binds to the exposed ends of the damaged DNA. Afterwards, the MRN complex recruits and activates the ATM kinase through its autophosphorylation at serine 1981 (Lee and Paull, 2005). Once activated, ATM phosphorylates a large number of its downstream targets (Matsuoka et al., 2007) that may further initiate the cell-cycle arrest, DNA repair, or apoptosis – the type of response depends on the extent and duration of DNA damage. Several of these targets, including p53 or Chk2 function as tumor suppressors *in vivo*, and their phosphorylation after the DNA damage event is critical. In general, phosphorylation of Chk2 at threonine 68 by ATM leads to its activation

and transmission of the checkpoint signal. The best-known example of checkpoint maintenance is the contribution of the tumor suppressor p53 and its transcriptional target $p21^{waf1/cip1}$ (p21) to cell cycle arrest. When p53 is activated through its phosphorylation at serine 15 by ATM/ATR, it binds to the promoters of multiple target genes and modulates their transcription (Fiscella et al., 1993). One of these genes is the cyclin-dependent kinase inhibitor p21 (El-Deiry et al., 1994), which inhibits the cyclin-E/cdk2 and cyclin-A/cdk2 complexes, and prevents G1/S transition. ATM-Chk2-p53-p21 cascade (Figure 4) then forms one of the key elements that regulates the cellular DDR, and defends the cell from the malignant transformation. Increasing autophosphorylation of ATM, phosphorylation of Chk2 and the overall activation of many other DDR proteins was observed in the early-stage tumors, suggesting the role of DDR as a barrier to the malignant progression of tumors (Bartkova et al., 2005; Gorgoulis et al., 2005).

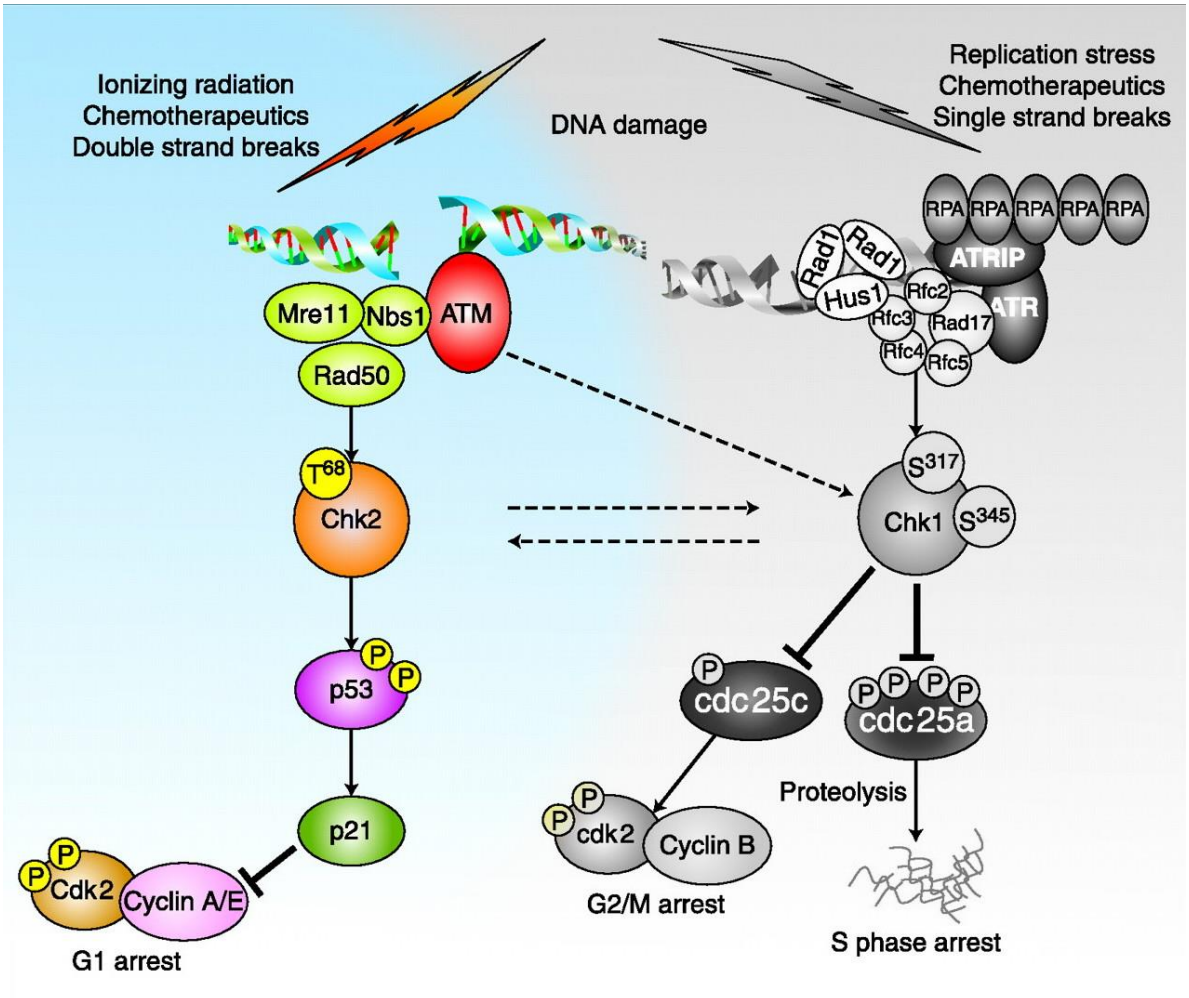


Figure 4. DDR pathways. ATM and ATR kinases respond to DNA damage by the phosphorylation and activation of the serine/threonine checkpoint kinases Chk1 and Chk2. These checkpoint kinases are transducers of the DNA damage signal and both phosphorylate a number of substrates involved in the DDR. The G_1 arrest, evolving after DSB induction, is modulated primarily by the ATM-Chk2-p53-p21 pathway (Ashwell and Zabludoff, 2008 – adjusted).

2.2.3. Cell senescence

Nearly all of normal mammalian somatic cells possess only a limited capacity of their replicative life *in vitro*. After the proliferative phase, the non-proliferative phase termed cellular senescence, arises. This phenomenon was first observed more than 50 years ago, when Hayflick and Moorhead showed that normal human fibroblasts did not proliferate indefinitely *in vitro* (Hayflick and Moorhead, 1961).

The cellular senescence is described as an essentially irreversible growth arrest. That can occur naturally by the telomere dysfunction, as the telomeres, the regions of repetitive nucleotide sequences at each end of the chromatid, are covered by proteins inhibiting various DNA repair machineries (Gorgoulis et al., 2005). Second, senescence may be caused also by mutation of oncogenes (Lee et al., 1999; Serrano et al., 1996) or it arises in the response to anti-cancer genotoxic therapies, as they are source of unrepaired DNA damage (Michishita et al., 1999). However, besides above mentioned stimuli for the senescent development, also other mechanisms were described (see Figure 5).

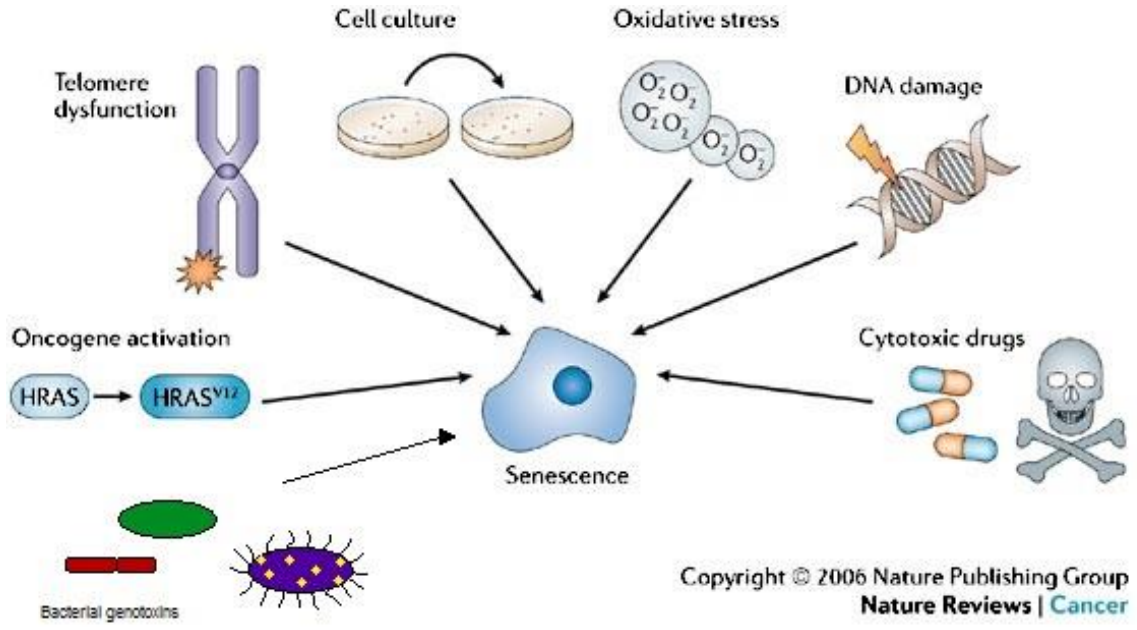


Figure 5. Senescence-inducing agents (Collado and Serrano, 2006 - adjusted).

The master regulators of senescence are p53 and protein retinoblastoma (Rb) together with their downstream targets p21 and p16, whose activation is essential and sufficient to induce senescence (McConnell et al., 1998). DDR caused by DNA damage engages primarily in the p53-p21 pathway, which causes the arrest of the cell cycle in G1 phase. Persistent DDR signaling then initiates the permanent senescence growth arrest (Fumagalli et al., 2014).

Senescent cells do not proliferate, but are still metabolically active and show widespread changes in the gene expression and cellular morphology. Senescent cells exhibit enlarged shape with flattened morphology and possess various nuclear abnormalities (multinucleation, polyploidy, etc.) (Dell'Orco and Whittle, 1994). Senescent cells also secrete numerous cytokines, chemokines, growth factors and proteases with autocrine and paracrine activities termed collectively called the senescence-associated secretory phenotype (SASP). SASP can be either beneficial or deleterious, depending on the physiological context and cytokines produced (for example, induction of inflammation or epithelial-to-mesenchymal transition (Ansieau et al., 2008)). A commonly used marker for senescent cells is based on histochemical staining for senescence-associated β-galactosidase activity (SA-β-gal) (Dimri et al., 1995), whose overexpression is derived from the expansion of lysosomes (Lee et al., 2006a).

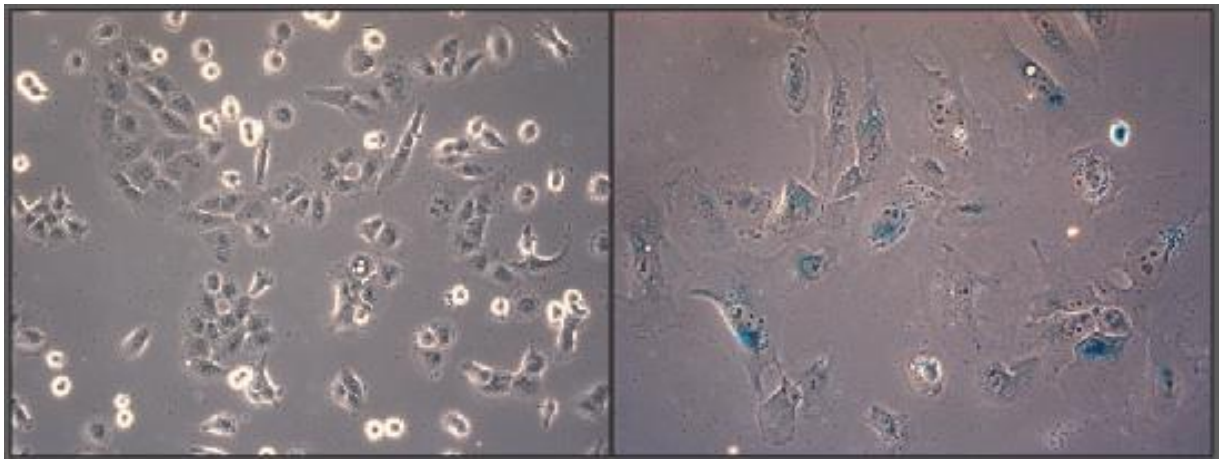


Figure 6. Senescence-associated β-galactosidase activity in senescent cells. Left – control, non-treated DU145 PCa cells; right – DU145 cells treated 5 times with 50 μM BrdU to induce senescence. SA-β-gal staining was used to indicate senescent cells (blue). Typical senescent phenotype is shown.

Most cancer cells differ from normal cells by the acquisition of immortality, meaning they do not have a finite replicative life span (Edington et al., 1995). The permanence of the proliferation arrest in senescent cells suggests that this mechanism serves as a barrier against cancer development (Sager, 1991). Nevertheless, the molecular changes, for example the inactivation of certain tumor suppressor genes (p53 or Rb), can lead to the re-entering to the cell cycle and eventually, to the proliferation with defective genome. It was estimated that the functional p53 is lost in more than a half of human cancers, thus becoming one of the most frequently mutated genes in the cancers (Brosh and Rotter, 2010). This forms a basis of the cancer progression.

2.3. Cancer metastasis and epithelial-to-mesenchymal transition

The most dangerous phase of the malignant tumor progression is the formation of metastases that causes about 90% of cancer death (Weigelt et al., 2005). Dissemination of the tumor cells to distant sites is a multistep process, whose proper mechanism remains poorly understood. In general, the metastatic process is divided into five distinct steps: local invasion, intravasation, transport through the circulatory system, extravasation and colonization (for a review, see (Shibue and Weinberg, 2011)). The local invasion is the first key step, when epithelial tumor cells lose their cell-to-cell contacts, become motile and leave the site of the primary tumor to invade nearby tissues. Then, during intravasation, tumor cells invade across the endothelial lamina and penetrate the walls of blood vessels and/or lymph nodes to enter the systemic circulation. In the course of circulation, only a small number of tumor cells survive the anchorage-independent growth conditions. During the extravasation, surviving cells may attach the vascular lumen at the distant sites of the body and infiltrate into the distant organs. Finally, during colonization, usually only a small fraction of neoplastic cells survive in the new stromal environment and establish micrometastasis with the proliferative potential (Shibue and Weinberg, 2011).

It is believed that the invasion, as the initial step of a metastasis, has a critical role in this process. Previous studies have shown that the developmental program termed epithelial-mesenchymal transition (EMT) plays a key role in promoting the metastasis in epithelium-derived carcinomas (Lee et al., 2006b; Xue et al., 2003). This suggests that understanding EMT and consequently metastasis is crucial for the future development of the novel strategies for the cancer treatment.

Epithelial cells are tightly linked together via several types of cellular junctions, including adherent junctions, tight junctions and desmosomes forming the layer which interacts with the basement membrane to maintain apical-basal polarity. While epithelial cells are polarized and carry out tissue specific functions, mesenchymal cells mostly play just a supporting role in the tissue and embed themselves inside the extracellular matrix. EMT is a process when epithelial cells undergo the morphological and molecular changes to acquire the mesenchymal-like properties. Transformation from epithelial to mesenchymal phenotype therefore leads to the enhanced migratory potential, resistance to apoptosis and invasiveness (Tiwari et al., 2012). The basic molecular mechanism of EMT is repression of adhesion molecules typical for the epithelium and subsequent acquisition of the mesenchymal markers.

The most common epithelial marker is the epithelial cadherin (E-cadherin) (Fleming et al., 2000). Among other epithelial markers belong also claudins, cytokeratin, occludins, laminin-1, desmoplakin, mucin 1, etc. The mesenchymal markers are fibronectin, vitronectin, vimentin, N-cadherin, FSP1, etc. (see Figure 7) After the cells go through the EMT process, they are able to differentiate into other cell types or revert back to the epithelial cells through a reverse process called mesenchymal-epithelial transition (MET) (Davies, 1996).

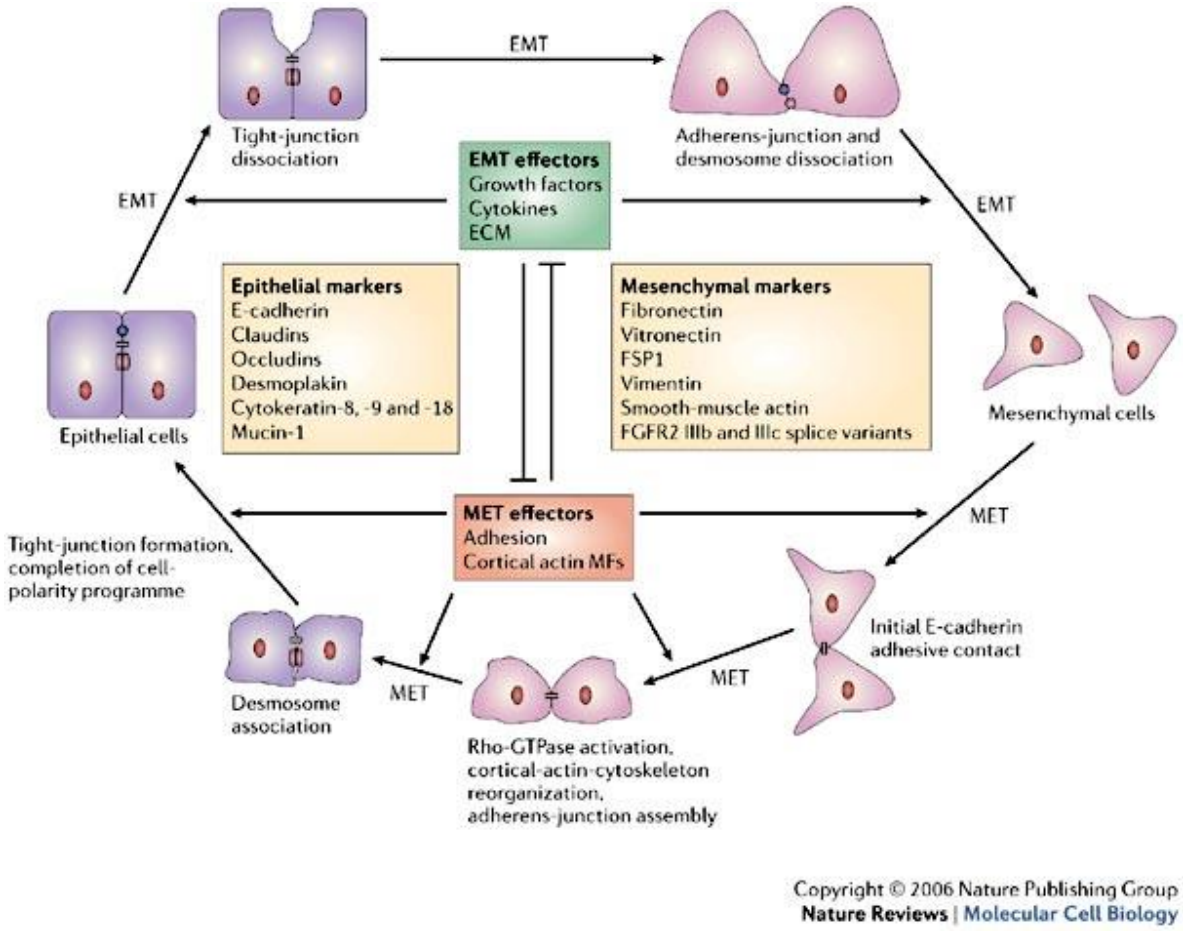


Figure 7. The cycle of events during which the epithelial cells are transformed into the mesenchymal cells and *vice versa*. The different stages during EMT (epithelial–mesenchymal transition) and the reverse process MET (mesenchymal–epithelial transition) are regulated by the effectors of EMT and MET, which influence each other. Important events during the progression of EMT and MET, including the regulation of the tight junctions and the adherent junctions, are indicated. E-cadherin (Epithelial cadherin), ECM (Extracellular matrix), FGFR (Fibroblast-growth-factor receptor-2), FSP (Fibroblast-specific protein-1), MFs (Microfilaments) (Adopted from (Thiery and Sleeman, 2006)).

The EMT was first described in 1995 as "epithelial-mesenchymal transformation" using a model of chick primitive streak formation, when EMT program was observed to allow stationary epithelial cells to gain the ability to migrate and invade during the developmental

morphogenesis to form one of the three embryonic layers, the mesoderm (for a review, see (Hay, 1995)). After some time the term "transformation" has been replaced by "transition".

Nowadays, EMTs are divided into three types with very different developmental consequences. The first type of EMT is very important in embryonic and organ development in most metazoans. For example, this process is involved in the formation of three-layered embryo by gastrulation (see above), or it also initiates placenta formation and organogenesis. Furthermore, EMT relates with wound healing, tissue regeneration and organ fibrosis, which represents the second type of EMT. Within fibrosis, the EMT associates with the inflammation, in the extreme cases continuing to organ destruction. Recently increasing number of studies strongly suggest that EMT program initiation is involved in the carcinoma progression and metastasis (for reviews, see (Huber et al., 2005; Hugo et al., 2007; Tsai and Yang, 2013)). Thus, various carcinoma cells are believed to undergo the last type of EMT, which shares many morphological and molecular features similar to those of the developmental EMT. These cells then lose their epithelial characteristics and may invade other tissues and organs. This is a basis of the metastasis, life-threatening manifestation of the cancer progression.

2.4. Transcription factor Snail

2.4.1. Structure and function

Snail, a member of the Snail superfamily of zinc-finger transcription factors, was first described in *Drosophila melanogaster* in 1984 (Grau et al., 1984). Snail was shown to be essential for the formation of the mesoderm during the embryo gastrulation. Later on, Snail homologues have been found in many species from invertebrate to vertebrate, including humans (Paznekas et al., 1999). In vertebrates, three Snail family members have been identified: SNAI1 (Snail), SNAI2 (Slug), and SNAI3 (Smuc).

All Snail family members serve often as transcriptional repressors, characterized by a common protein organization (Figure 8). They share a highly conserved C-terminal domain, which contains four to six zinc fingers of the C_2H_2 type. The zinc fingers mediate the sequence-specific interactions with DNA by binding to the consensus binding sites in its target gene promoters. This motif represents a subset of the E-box that contains the consensus sequence 5´-CANNTG-3´. The C-terminus is responsible for the repressor activity of the Snail. The N-terminal of the Snail family members is less conserved. However, all the vertebrate members contain the evolutionary conserved SNAG (for Snail/Gfi) domain, which is required for Snail interaction with several transcriptional corepressor complexes (Figure 8). The central part of Snail comprises of a nuclear export sequence (NES), controlling the subcellular localization of Snail, and a serine-rich domain (SRD), modulating the Snail protein stabilization (Franco et al., 2010). The central region of the protein is also responsible for Snail activity, as it contains most sites for the post-translation modification.

Snail expression and activity can be regulated by various factors at the transcription as well as post-translation level. Localization of Snail in the cytosol potentiates its ubiquitination and its subsequent proteasomal degradation, while the accumulation of Snail in the nucleus promotes its transcriptional function. The main regulator of Snail subcellular localization is glycogen synthase kinase-3β (GSK-3β), which phosphorylates Snail and thus promotes its export from the nucleus and the degradation in the cytosol (Zhou et al., 2004). Moreover, the various signals from the tumor microenvironment, including soluble growth factors and cytokines, may regulate Snail subcellular localization and activity, especially through its phosphorylation (Peinado et al., 2003; Wu et al., 2009).

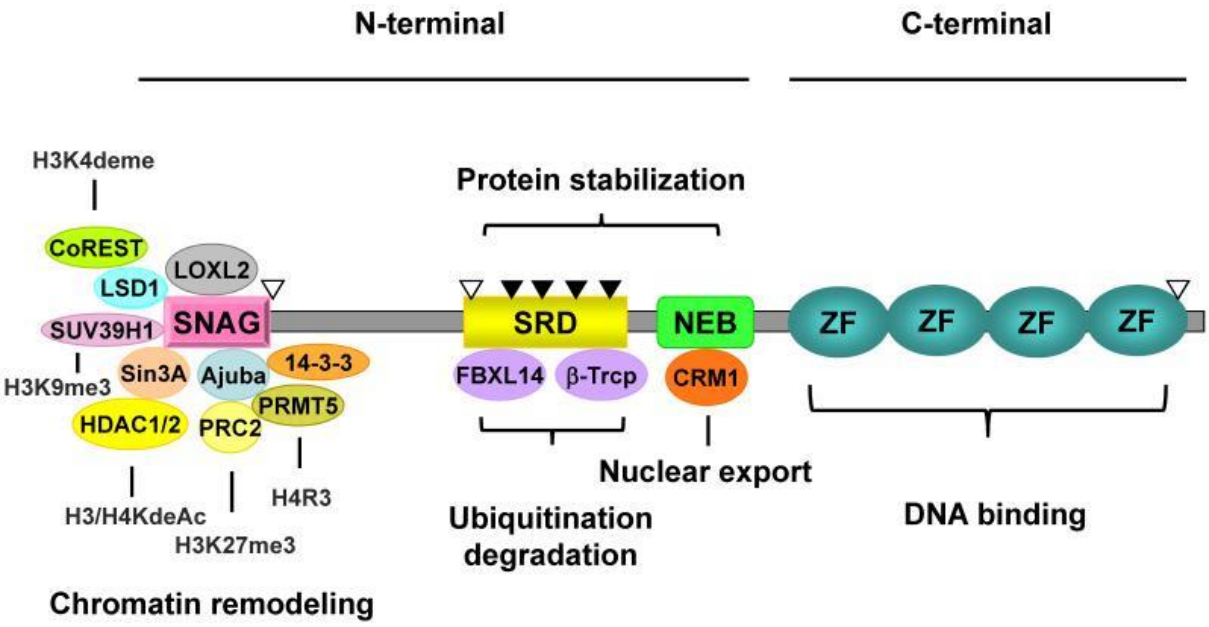


Figure 8. Snail contains an N-terminal SNAG domain and C-terminal zinc finger domains (ZF). The N-terminal SNAG domain interacts with several co-repressors and epigenetic remodeling complexes, and the C-terminal zinc finger domains are responsible for DNA binding. The serine-rich domain (SRD) and nuclear export sequence (NES) control Snail protein stability and subcellular localization. Phosphorylation sites are indicated as triangles (Adopted from (Wang et al., 2013)).

Snail has a crucial role in mesoderm formation. As its deficiency in mouse embryos leads to the defective formation of mesoderm (Carver et al., 2001) and elevated Snail levels were observed in metastatic lesions in various types of tumors (Henderson et al., 2015; Jin et al., 2010; Yang et al., 2007), Snail is characterized as a master regulator of EMT.

2.4.2. Role of Snail in epithelial-mesenchymal transition

EMT can be induced by multiple factors, such as growth factors – transforming growth factor β (TGF-β), epidermal growth factor (EGF); and transcription factors Snail, Twist1/2, Slug, etc. (Ciruna and Rossant, 2001; Lu et al., 2003; Peinado et al., 2003). Earlier studies have shown that the adherent junction proteins (like E-cadherin and occludins), intermediate filaments (like cytokeratins) and desmosomes (like desmoplakins) are repressed in the epithelial cells during EMT. This is accompanied by the synthesis of mesenchymal markers such as vimentin or N-cadherin and gaining the flattened phenotype. Afterwards, the cells detach from the basement membrane and become more migratory (reviewed by (Boyer et al., 2000)).

As mentioned above, transcription factor Snail can mediate EMT through the down-regulation of the cell adhesion molecules and the tight junction proteins by binding to

E-box sequence located in the promoter region of its target genes. A hallmark of EMT, caused by the Snail overexpression, is the reduction of transcription of cell-to-cell adhesion molecule E-cadherin (Cano et al., 2000). E-cadherin is an important keeper of the epithelial phenotype, whose decrease is systematically observed at the sites of EMT during the development and even during cancer dissemination (Batlle et al., 2000). Snail expression also leads to the repression of several epithelial and tight junction genes that encode cytokeratin 18, mucin 1 (Guaita et al., 2002), claudins, occludins (Ikenouchi et al., 2003) or zona occludin-1 (Ohkubo and Ozawa, 2004), i.e. proteins that promote the EMT. In addition, Snail can mediate an increase in the expression of some mesenchymal markers like vimentin, fibronectin, matrix metalloproteinases (MMPs) and RhoA (Zhang et al., 2005) and can also induce other E-cadherin repressors such as Zeb-1 and Zeb-2 (Takkunen et al., 2006).

On the other hand, as the EMT is triggered by the stimuli from its surroundings, many secreted soluble factors such as transforming growth factor-β (TGF-β), epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), vascular endothelial growth factor-A (VEGF-A), tumor necrosis factor-α (TNF-α) or Wnt ligands/pathway can regulate Snail expression, stabilization and activity to promote EMT. This means that, for example, TGF-β – a multifunctional cytokine and the overall regulator of EMT, can up-regulate Snail and promotes its nuclear localization and Snail then mediates the escape from the tumor suppressive effects of TGF-β in the late stages (Franco et al., 2010).

As a critical regulator of multiple signaling pathways leading to EMT, the Snail expression is closely associated with the cancer metastasis. The involvement of Snail in tumor progression is supported by its expression in invasive carcinoma cell lines (Cano et al., 2000) and by the graded expression of Snail in biopsies from patients with various types of carcinoma – breast cancer (Blanco et al., 2002), gastric cancer (Rosivatz et al., 2002) or hepatocellular cancer (Sugimachi et al., 2003). When comparing to the normal tissue, Snail expression was also observed to increase in the localized and further in the metastatic PCa (Dhanasekaran et al., 2001). Moreover, the knock-down of Snail significantly inhibits tumor growth and metastasis by increasing tumor-infiltrating lymphocytes and the systemic immune responses (Kudo-Saito et al., 2009). Together with the fact that Snail is associated with the tumor recurrence and the resistance to chemotherapy and radiotherapy (Kyjacova et al., 2015), Snail seems to be an effective target for preventing the cancer metastases.

2.4.3. Role of Snail in DNA damage

Although a lot is known about the role of Snail in EMT and the connection between Snail expression and metastases becomes a frequent subject of the research, we do not know much about the role of Snail in the DNA damage response.

ROS represent DNA damaging agents produced spontaneously during the normal cellular metabolism. Besides, ROS are also released in the tumor tissue, which positively correlates with the clinical stage in the small cell lung cancer and squamous cell carcinoma patients (Zieba et al., 2000). Furthermore, it has been reported that antioxidant manganese superoxide dismutase enzyme levels are lower, but nuclear oxidative damage products are higher in metastatic tissue of PCa, compared to the primary tissue (Oberley et al., 2000). This suggests that the increase in ROS levels due to the repression of the antioxidants may contribute to the DNA damage and development of PCa. EMT was also observed to associate with the increased ROS and Snail and abrogation of ROS may inhibit EMT (Radisky et al., 2005). Another study also shows that PCa cell line model ARCaP established by overexpressing Snail displayed increased ROS *in vitro* and *in vivo* in mouse (Barnett et al., 2011). Finally, ROS has been shown to induce the Snail expression in breast cancer cells and conversely Snail can upregulate ROS and therefore induce EMT (Barnett et al., 2011).

The essential study, describing a relationship between DDR and Snail expression, shows that ATM, the main regulator of response to DSBs, can phosphorylate Snail on serine 100 and therefore mediate its stabilization (Sun et al., 2012). It was proposed that Snail phosphorylation by ATM leads to its resistance to GSK-3β-mediated degradation in cytosol and accumulation of Snail in the nucleus. Thus, ATM-mediated Snail stabilization in response to IR is essential to regulate cellular radiosensitivity (Boohaker et al., 2013).

Despite the well-characterized role in EMT and cancer metastasis, it is less clear whether and how Snail might be involved in DDR pathways and checkpoint control maintenance.

3. Aims of the study

Widely used anti-cancer drugs, together with radiation treatment, are the best-known sources of the DNA damage. Recently have been published several studies dealing with the relationship between the genotoxic cancer treatment and the increasing induction of metastasis (Camphausen et al., 2001; Su et al., 2012; Volk-Draper et al., 2014). The EMT is considered as the founding stone of cancer metastasis and the transcription factor Snail is believed to be one of the main mediators of EMT specific for human cancer. Moreover, it has been shown that Snail has a role also in the processes of radioresistance and chemoresistance of surviving cancer cells.

In the light of these data, we wanted to test whether there is an interaction between DNA damage response and expression of Snail in PCa. Therefore, for the purposes to study this mechanism, we have identified three specific aims to be examined in this thesis.

1. Testing role of ATM in regulation of Snail stabilization in response to DNA damage

2. Deciphering the role of Snail in the expression of Cdk inhibitor $p21^{waf1/cip1}$

3. Identifying novel Snail transcriptional targets involved in DDR

4. Material and methods

4.1. Chemicals and other material

Chemicals, etc.	Manufacturer, Country
10 mM dNTPs (deoxynucleotide triphosphates)	Fermentas International Inc., USA
2-Buthanol	Penta, CR
Acetic acid	Penta, CR
Acrylamide/Bis	Serva Electrophoresis GmbH, Germany
APS (ammonium persulfate)	Sigma-Aldrich, USA
Aqua pro injectione	B. Braun, Germany
Bromphenol Blue	Lachema, CR
Trisodium citrate dihydrate	Sigma-Aldrich, USA
DAPI, 4',6-diamidino-2-phenylindole	Sigma-Aldrich, USA
Dithiothreitol (DTT)	Sigma-Aldrich, USA
DMEM (Dulbecco's Modified Eagle's Medium)	IMG ASCR, v.v.i., CR
DMSO (Dimethylsulphoxide)	Sigma-Aldrich, USA
Double-distilled sterile H_2O	IMG ASCR, v.v.i., CR
EDTA (Ethylenediaminetetraacetic acid)	IMG ASCR, v.v.i., CR
Ethanol	Penta, CR
Fetal Bovine Serum (FBS)	Life Technologies, USA
Formaldehyde	Sigma-Aldrich, USA
FuGENE® 6 Transfection reagent	Roche Applied Science, Germany
Glycerol	Sigma-Aldrich, USA
KU55933 ATM kinase inhibitor	Calbiochem, Switzerland
Lipofectamine™ RNAiMAX	Invitrogen, USA
Medical X-ray film Blue	AGFA HealthCare, Belgium
Methanol	Penta, CR
β-Mercaptoethanol (2-Mercaptoethanol)	Sigma-Aldrich, USA
Na_2HPO_4 (Sodium phosphate anhydrous)	Sigma-Aldrich, USA
NaN_3 (Sodium azide)	Koch-Light Laboratories Ldt. UK
Negative control siRNA	Applied Biosystems, USA

Nonfat dry milk	Novako, CR
PageRuler prestained protein ladder # 26616	Fermentas International Inc., USA
PBS (Phosphate buffered saline)	IMG ASCR, v.v.i.; CR
PonceauS	Fluka, Switzerland
Pure Nitrocellulose Blotting Membrane	Pall Corporation, USA
RNase Inhibitor	Fermentas International Inc., USA
RPMI (Roswell Park Memorial Institute)	IMG ASCR, v.v.i.; CR
SDS (Sodium dodecyl sulfate)	Serva Electrophoresis GmbH, Germany
Snail siRNA	Applied Biosystems, USA
SYBR Select Master mix	Life technologies, USA
TaqMan reverse transcription reagent	Life technologies, USA
TEMED (N,N,N',N'-tetramethylethylendiamine)	Fluka, Switzerland
Trypsin/EDTA (Ethylenediaminetetraacetic acid)	IMG ASCR, v.v.i.; CR
Tween-20	Sigma-Aldrich, USA
Triton X-100 (polyethylene glycol tertoctylphenyl ether)	Fluka, Switzerland
Tris (Trishydroxymethylaminomethane)	Serva Electrophoresis GmbH, Germany
TGS buffer 10 x (192 mM glycine, 25 mM Tris, 0.1% (w/v) SDS, pH 8.3)	Bio Rad, USA
TG buffer 10 x (192 mM glycine, 25 mM Tris, pH 8.3)	Bio Rad, USA
VECTASHIELD HardSet Mounting	Vector Laboratories, USA
X-gal (5-Bromo-4-Chloro-3-Indolyl beta-D-galactopyranoside, 98%)	Sigma-Aldrich, USA

4.1.1. Kits and pre-designed systems

Pre-designed system, Country

ECL Western Blotting System, Amersham, USA
BCA Protein Assay, Thermo Scientific, USA
Rneasy Mini Kit, Quiagen Sciences, Germantown, MD, USA
High Capacity cDNA Reverse Transcription kit, Foster city, CA, Applied Biosystems, USA

4.1.2. Instruments

Manufacturer, Country

7300 Real-Time ABI Prism PCR System; Applied Biosystems, Foster City, CA, USA
Analytical weights AE 240; Mettler, USA
Modulus™ Microplate Multimode reader; Turner Biosystems, USA
Mini PROTEAN® 3 Cell wet tank system; Bio Rad, USA
BioSafety Cabinet Bio-II-A Telstar, Spain
Bürker counting chamber; Laboroptik, Germany
Centrifuge 5415R; Eppendorf, Germany
Centrifuge 5424; Eppendorf, Germany
Centrifuge NF400; Nüve Inc.,Turkey
CO_2 Incubator FORMA Series II Water Jacket; Thermo Fisher Scientific Inc., USA
Leica DM6000 fluorescent microscope; Leica microsystems, Zeiss, Germany
Leica DM IL, inverted contrasting microscope; Leica Microsystems, Zeiss, Germany
Microplate photometer Multiskan® EX; Thermo Fisher Scientific Inc., Waltham, USA
Minicentrifuge Z 100; Hermle LaborTechnik GmbH, Germany
NanoDrop® ND-1000 Spectrophotometer; Thermo Fisher Scientific Inc., USA
PIPETMANs Neo® Set; Gilson Inc., Middleton, USA
SDS-PAGE Apparatus Mini-PROTEAN Tetra Cell; Bio Rad, USA
Soniprep 150 ultrasonic disintegrator; MSE, London, UK
T-200 X-ray instrument; Wolf-Medizintechnik, St. Gangloff, Germany
Thermomixer comfort; Eppendorf, Germany
Vortex Lab dancer; VWR, Germany
Water bath BM402; Nüve Inc., Turke

4.1.3. Antibodies

Primary antibodies	Manufacturer, Country
rabbit monoclonal anti-Snail	Cell Signaling, ♯3879, Biotech, Praha, CR
mouse monoclonal anti-p21	Santa Cruz, sc-56335, Heidelberg, Germany
mouse monoclonal anti-γ-tubulin	gift from Pavel Draber, IMG, CR
rabbit polyclonal anti-phosphorylated threonine 68 Chk2 (Chk2pT68)	Cell Signaling, ♯2661, Biotech, Praha, CR
mouse monoclonal anti-Chk2	Millipore, 05-649, MA, USA
rabbit polyclonal anti-phosphorylated serine 15 p53 (p53pS15)	Cell Signaling, ♯9284, Biotech, Praha, CR
mouse monoclonal anti-p53	Santa Cruz, sc-126, Heidelberg, Germany
mouse monoclonal anti-GAPDH	GeneTEX, GTX30666, USA
mouse monoclonal E-cadherin	Santa Cruz, sc-8426, Heidelberg, Germany
rabbit polyclonal anti-p16INK4a	Santa Cruz, sc-759, Heidelberg, Germany
mouse monoclonal anti-pRb	554136, BD Pharmingen, Heidelberg, Germany

Secondary antibodies	Manufacturer, Country
horseradish peroxidase-conjugated goat anti-mouse	Bio-Rad, Hercules, CA, USA
horseradish peroxidase-conjugated goat anti-rabbit	Bio-Rad, Hercules, CA, USA
anti-mouse IgG antibody Alexa 488	Carlsbad, CA, USA
anti-rabbit IgG antibody Alexa 568	Carlsbad, CA, USA

4.2. Methods

4.2.1. Cell lines and growth conditions:

Thawing cells

Cells in cryovials, previously stored in liquid nitrogen, were warmed by placing the tube directly from the liquid nitrogen container into a 37°C water bath with moderate shaking. As soon as the last ice crystal was melted, the cells were immediately diluted into pre-warmed DMEM medium and centrifuged at 400 x g for 5 minutes. Supernatant was removed and cells were resuspended in fresh DMEM.

Cell culture

All manipulations with the cells were performed in the sterile atmosphere of laminar flow box (BioSafety Cabinet Bio-II-A Telstar, Spain). Used equipment was sterile, autoclaved or disposable.

Human PCa cell lines DU145, PC-3, LNCaP, human breast carcinoma cell line MCF-7, human embryonic kidney cell line HEK293 and human colorectal carcinoma cell lines HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in appropriate medium - Roswell Park Memorial Institute (RPMI) (in case of LNCaP) or Dulbecco's modified Eagle's medium (DMEM) (all remaining lines) supplemented with 10% fetal bovine serum (FBS) and antibiotics penicillin (100 U/ml) and streptomycin (100 ng/ml). Cells were grown at 37°C under 5% CO_2 atmosphere and 95% humidity. Cells were replated according to their growth rate, usually when reached about 90% confluence.

Counting cells

Bürker counting chamber was used for estimating cell counts, accordingly to the mammalian cell culture protocol (Freshney and Freshney, 2005).

4.2.2. Treating cells

To induce DNA damage, cells were irradiated with single dose (2, 5 or 10 Gy) or multiple doses (2 Gy) applied daily using T-200 X-ray instrument (Wolf-Medizintechnik, St. Gangloff, Germany). Alternatively, DNA damage was induced by genotoxic drugs camptothecin (CPT; 2 μM for 2 or 3 hours; Sigma, C9911; (Avemann et al., 1988) or neocarzinostatin (NCS; 1:5000; Sigma, N9162; (Ishida et al., 1965)). KU55933 (10 μM, Calbiochem, #118500; (Hickson et al., 2004) was used 1 h before other treatments to inhibit ATM kinase.

4.2.3. SDS-PAGE and Western blotting analysis

Cells were washed with PBS, lysed in Laemmli SDS sample lysis buffer (2% SDS, 50 mM Tris-Cl, pH 6.8, 10% glycerol in double distilled H_2O) and sonicated for 3 x 15 seconds at 3 microns of amplitude with 15 seconds cooling intervals on Soniprep 150 (MSE, London, UK). Concentration of proteins was estimated by the bicinchoninic acid assay (BCA) (Pierce Biotechnology Inc., Rockford, USA) accordingly to the manufacturer's protocol. 100 mM DTT and 0.01% bromphenol blue was added to lysates, which were then denaturated at 96°C for 1 minute before separation by SDS-PAGE (12 and 14% acrylamide gels were used). The same protein amount (20 - 40 μg) was loaded into each well. Proteins were electrotransferred onto a nitrocellulose membrane (Amersham™ Hybond ECL, GE Healthcare Life Sciences) using wet transfer and detected by specific antibodies combined with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit, goat anti-mouse, Bio-Rad, Hercules, CA, USA). Peroxidase activity was detected by ECL (Pierce Biotechnology Inc.). GAPDH or γ-tubulin was used as a marker of equal loading.

Efficiency of protein transfer was checked via staining the total proteins on the membrane using PonceauS (Fluka, Switzerland) The membrane was washed in PBS and blocked in 5% non-fat milk at the room temperature (RT) for 1 hour. Membrane was then incubated overnight at 4°C with primary antibody diluted in PBS/Tween-20 with 1% non-fat milk. After incubation the membrane was washed three times for 5 minutes in PBS/Tween-20, incubated with the appropriate HRP-conjugated secondary antibody diluted in PBS/Tween-20 with 2.5% non-fat milk for 1 h and then again washed three times in PBS/Tween-20 for at least 5 minutes each wash.

Components for 10 ml separating SDS-PAGE gel

Acrylamide percentage	**12%**	**14%**
H_2O distilled	3.2 ml	2.2 ml
Acrylamide/Bis 30%	4 ml	5 ml
SDS electrophoresis buffer 1 *	2.7 ml	2.7 ml
Ammonium persulfate (APS) 10%	100 µl	100 µl
TEMED	10 µl	10 µl

Components for 5 ml stacking SDS-PAGE gel

Acrylamide percentage	**5%**
H_2O distilled	2.975 ml
Acrylamide / Bis 30%	1.25 ml
SDS electrophoresis buffer 2 **	0.72 ml
Ammonium persulfate (APS) 10%	50 µl
TEMED	5 µl

*SDS electrophoresis buffer 1 (1.5 mM Tris, 0.4% SDS, pH 8.8)

**SDS electrophoresis Buffer 2 (0.5 mM Tris, 0.4% SDS, pH 6.8)

4.2.4. Indirect immunofluorescence

Cells grown on sterile glass coverslips were washed with PBS and fixed by 4% formaldehyde for 15 minutes at RT and permeabilized by 0.1% Triton X-100 for 15 min at RT. To block unspecific signals cells were incubated in 10% FBS for 30 minutes.

For immunofluorescence staining, fixed cells were incubated with primary antibodies diluted in PBS for 1 hour at RT under humidity controlled conditions and then extensively washed with PBS. The incubation with secondary antibodies was performed for 1 hour at RT in PBS. Nuclei were counterstained with 4',6-diamidino-2-phenylindole staining (DAPI; Sigma, St. Louis, MO, USA) followed by mounting in Mowiol (Sigma, St. Louis, MO, USA) or Vectashield HardSet Mounting Medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). Images were captured by fluorescent microscope Leica DM6000 (Leica Microsystems, Zeiss, Germany) equipped with monochrome digital camera DFC350 FX and Leica LAS AF Lite software.

4.2.5. Quantitative real time RT-PCR (qRT-PCR)

Cells were washed with PBS and lysed in RLT lysis buffer (RNeasy Mini Kit, Quiagen Sciences, Germantown, MD, USA). Total RNA samples were isolated using RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA) according to the manufacturer's instructions. 200 ng of isolated RNA was transcribed into cDNA with random hexamer primers using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster city, CA, USA). qRT-PCR was performed in ABI Prism 7300 (Applied Biosystems, Foster City, CA, USA) using SYBR Select Master Mix containing SYBR GreenE dye (Applied Biosystems, Foster City, CA, USA). Each sample was measured as technical triplicate. The relative quantity of cDNA was estimated by ΔΔCt method (Livak and Schmittgen, 2001). Data from PCR array were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and verified with the following set of primers (Sigma, St. Louis, MO, USA):

	Forward primer (5′→ 3′)	**Reverse primer (5′→ 3′)**
Snail	TGCCCTCAAGATGCACATCCGA	GGGACAGGAGAAGGGCTTCTC
p21	TCACTGTCTTGTACCCTTGTGC	GGCGTTTGGAGTGGTAGAAA
SSBP1	TCTGTCTGGGACGATGTTG	GTTTGGCTCACTGAAGTTAGG
CDH1	TGAAGGTGACAGAGCCTCTGGAT	TGGGTGAATTCGGGCTTGTT
GAPDH	GTCGGAGTCAACGGATTTGG	AAAAGCAGCCCTGGTGACC

4.2.6. siRNA interference-mediated gene knock-down

Cells were seeded 1 day before transfection to be approximately 60-80% confluent at the time of transfection. Transfection was performed according to manufacturer's instructions using Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA). The sequence of the Snail siRNA (siSN, siSnail) was 5-GAA UGU CCC UGC UCC ACA Att. Non-targeting siRNA sequences (siNC) were used as a negative control siRNA. All siRNAs were purchased from Applied Biosystems (Foster City, CA, USA). 48 hours post transfection, cells were irradiated or treated as indicated.

4.2.7. Cell transfection for gene ectopic expression

Cell lines were transfected at 60-80% confluence with 0.5 µg/ml pEGFP-C2 plasmid (Addgene, USA) expressing Snail fused with GFP using FuGENE® 6 Transfection reagent (Roche Applied Science, Germany) according to the manufacturer's protocol. After at least 24 h, the number of cells expressing the GFP was checked by fluorescent microscopy.

5. Results

5.1. Testing the role of ATM in regulation of Snail stabilization in response to DNA damage

It was observed that Snail is involved in the induction of non-adherent growth, EMT and resistance to anoikis in response to fractionated irradiation (fIR) in PCa cell lines. (Kyjacova et al., 2015). This phenomenon was also described in other cancer cell lines (colorectal cancer (Hoshino et al., 2009), non-small cell lung carcinoma (Shintani et al., 2011), etc.) after different genotoxic insults (mostly chemotherapy treatment). To reveal a mechanistic link between genotoxic stress and above mentioned phenomena, we tested whether ATM kinase, activated by DNA damage, can directly phosphorylate and thus stabilize Snail (Figure 9), as was described recently (Sun et al., 2012). Using breast cancer cell lines MDM-MB-231 and MCF-7 Sun *et al.* showed that the presence of chemical inhibitor of ATM kinase KU55933 prevented Snail proteasomal degradation after either after IR or chemical stress (camptothecin, CPT, a topoisomerase I poison) (for CPT, see Figure 9). Moreover, they analyzed human invasive breast cancer tissues and found that protein level of Snail positively correlates with the level of activated ATM kinase, indicating a role of ATM in Snail regulation (Sun et al., 2012).

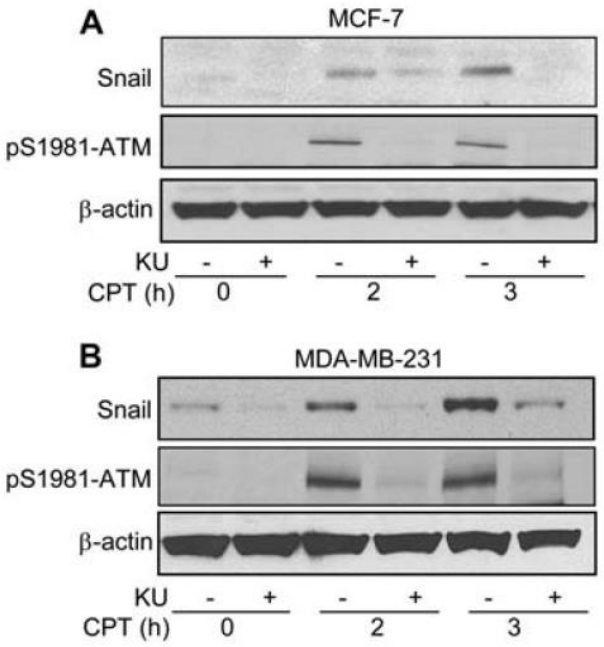


Figure 9. ATM regulates Snail stabilization in response to DNA damage. MCF-7 cells **(A)** or MDA-MB-231 cells **(B)** were pretreated with KU55933 (10 mM) for 1 h followed by CPT (2 mM) treatment for 2 or 3 h. Total cell lysates were collected and Snail, pS1981-ATM and β-actin were immunoblotted (Sun et al., 2012).

As radiotherapy is a common treatment for PCa and Snail is implicated in acquired resistance to both radiotherapy and chemotherapy (Kurrey et al., 2009), we decided to investigate the role of ATM in Snail stabilization after one dose of IR (10 Gy). We pretreated metastasis-derived PCa cell line DU145 and breast cancer cell line MCF-7 by ATM inhibitor KU55933 for 1 hour before IR and harvested the cells after 3 and 24 hours, respectively. Then we analyzed protein levels of Snail, Chk2 kinase phosphorylated at threonine 68 (Chk2pT68), Chk2 (Chk2 total) and GAPDH (loading control) by western blotting.

Since Chk2 is a direct substrate of ATM kinase and its phosphorylated form is one of the main markers of activated DDR pathway (Falck et al., 2001), we expected a significant decrease of Chk2pT68 protein levels in response to KU55933 treatment, notably after DNA damage induction.

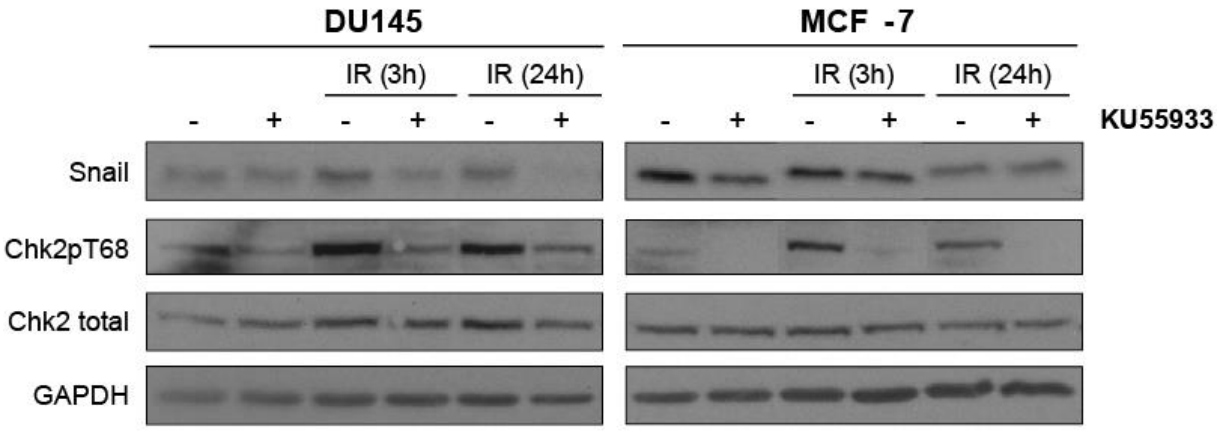


Figure 10. Effect of ATM inhibition on Snail stabilization after DNA damage induced by IR. Levels of Snail, Chk2 phosphorylated on threonine 68 (Chk2pT68), Chk2 total and GAPDH (loading control) in DU145 and MCF-7 cells after 1 h-pretreatment with KU55933 (10 μM) followed by irradiation (10 Gy) for 3 or 24 h. Represents pictures of 2 independent experiments are shown.

As shown in Figure 10, Chk2pT68 levels increased in KU55933-nontreated irradiated cells, which confirm the DNA damage induction. The level of Chk2pT68 was apparently reduced in KU55933-treated cells, indicating a proper function of ATM inhibitor. With regard to Snail protein level, we observed an obvious decrease of its level after the addition of KU55933 to subsequently irradiated DU145 cells indicating the role of ATM kinase in Snail stabilization upon IR in this PCa cell line. On the other hand, in case of MCF-7 breast adenocarcinoma cells Snail was degraded during inhibition of ATM only in control samples and samples harvested 3 h after IR. Furthermore, the Snail protein levels cells seemed rather to decline 24 hours after irradiation independently of ATM.

To achieve more conclusive results, we decided to follow exactly the same conditions mentioned in the study of Sun *et al.*, using (besides DU145) MCF-7 cells and CPT as a genotoxic agent. Cells were again pretreated for 1 hour with ATM inhibitor KU55933 and followed by CPT treatment for 2 or 3 hours. Total cells lysates were then prepared and analyzed for Snail, Chk2pT68, Chk2 total and GAPDH (loading control) by western blotting. As shown in Figure 11, CPT treatment caused high increase in the Snail level in both cell lines. The activity of ATM in KU55933-treated cells was reduced as indicated by diminished level of Chk2pT68. Provided ATM regulates Snail stabilization and Snail accumulation in the nucleus, there should be no up-regulation in the levels of Snail in KU55933-treated cells after CPT treatment. Nevertheless, increased degradation of Snail was observed after combined treatment with CPT and KU55933 inhibitor in DU145 only at one time-point (2 h). On the other hand in MCF-7 cells, the effect of ATM inhibition on Snail destabilization was detected in control cells and, in small extent, in samples treated with CPT for 3 h. Despite we observed ATM-mediated Snail stabilization in DU145 and MCF-7 cells undergoing IR, the resulting decrease of Snail level after ATM inhibition was not as clear as we expected, especially after treatment with CPT. However, to make final conclusion, more experiments are needed.

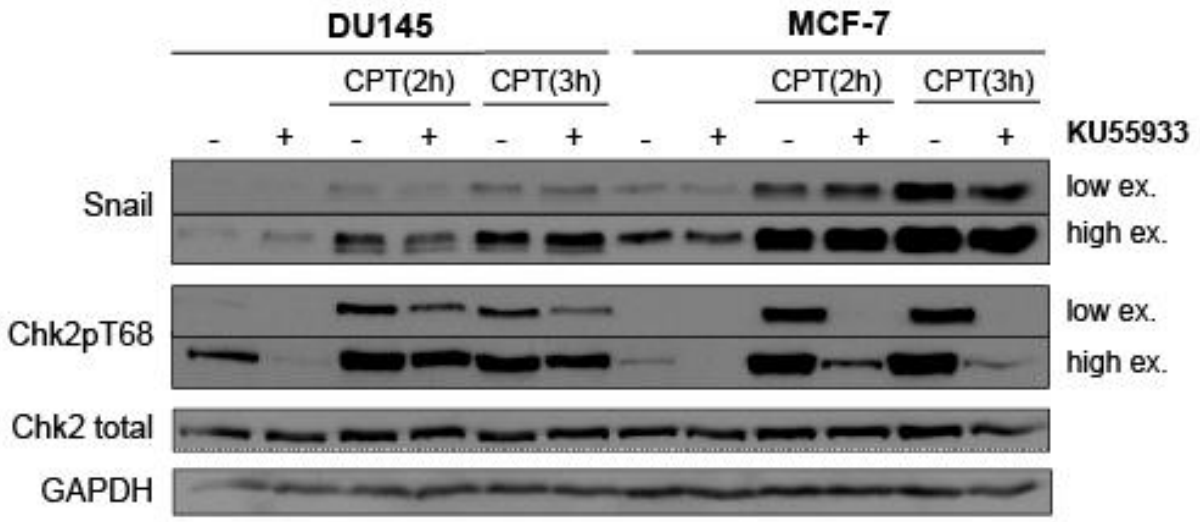


Figure 11. Effect of ATM downregulation on Snail stabilization after DNA gamage induced by CPT. Immunoblotting detection of Snail and Chk2 phosphorylated on threonine 68 (Chk2pT68) and Chk2 total protein (Chk2 total). GAPDH was used as a loading control. DU145 and MCF-7 cells were treated with ATM inhibitor KU55933 (10 μM; 1 h) followed by CPT (2 μM) treatment (2 or 3 h). Proper function of inhibitor is demonstrated by decreased level of Chk2pT68. Representative images of three independent experiments are shown.

5.2. Deciphering the role of Snail in expression of Cdk inhibitor p21$^{waf1/cip1}$

After DNA damage, multiple components of DDR machinery are activated in the cell, resulting in cell cycle arrest and DNA repair. An important part of DDR and subsequent regulation of the cell cycle is an activation of cell cycle checkpoint via ATM-p53-p21 pathway. The tumor suppressor p53 is directly phosphorylated by ATM kinase at serine 15 (Canman et al., 1998), which leads to its stabilization, tetramerisation and transcription activation. Cyclin-dependent kinase inhibitor p21 is one of the most important transcriptional targets of p53 involved in cell cycle regulation (Figure 4; (El-Deiry et al., 1994). p21 binds to and inhibits predominantly cyclin-A/Cdk2 and cyclin-E/Cdk2 complexes both orchestrating the progression through the cell cycle, resulting in the cell cycle arrest mainly in G1 phase (Stewart et al., 1999).

Although ATM/p53-mediated expression of p21 is the dominant pathway induced as a consequence of DNA double strand breaks, it was shown that p21 can be activated also by p53-independent pathways (Michieli et al., 1994) and several other mechanisms of p21 induction after genotoxic stress were identified in p53-negative background (Gartenhaus et al., 1996; Loignon et al., 1997). Importantly, it was shown that transcription factor Snail may negatively affect the expression of p21 in the human osteosarcoma MG63 cells (Takahashi et al., 2004).

As noted above, Snail binds to the 5´-CANNTG-3´ consensus motifs (E-boxes) in the promoters of its targets genes (Batlle et al., 2000; Pavletich and Pabo, 1991) and thus regulates its transcription. It was reported that knock-down of Snail by RNA interference downregulates p21 in MG63 cell line with mutated p53 (Takahashi et al., 2004) (Masuda et al., 1987), which is mediated via DNA binding of Snail to its consensus sequences present in p21 gene promoter (Kurrey et al., 2009). Moreover, ectopic expression of Snail in MDCK cells bearing wild-type p53 (Chen et al., 2006) resulted in overexpression of p21 (Vega et al., 2004). These facts led us to investigate the role of Snail in regulation of p21 during the genotoxic stress induced by IR.

For this purpose, we took advantage of the isogenic colorectal carcinoma cell line HCT116 p53 wild-type ($p53^{+/+}$) and null ($p53^{-/-}$) (Sur et al., 2009), which we irradiated and monitored from 0.5 to 48 hours (Figure 12). We noticed that Snail protein level at unperturbed conditions is significantly higher in $p53^{-/-}$ HCT116 compared to $p53^{+/+}$ cells. After the IR the level of Snail was peaking at hour 3 in $p53^{+/+}$ cells and then slowly declined until it reached control level as soon as 48 hours after IR. In contrast, the Snail level in $p53^{-/-}$ HCT116 cells remained unchanged until 3 h time-point after IR, and then had rather decreasing tendency in

comparison with non-irradiated sample. Compared to non-irradiated cells, p21 protein level gradually increased in irradiated cells after IR exposure in p53 wild type cells, however, we detected p21 only at 48 h time-point after IR in p53 null cells indicating low starting levels of this protein in the absence of its conventional transcriptional inducer p53. Together, Snail and 21 levels appeared to change in opposite direction, especially in p53 wild type cells. Chk2pT68 was used as the indicator of DDR activation with the peak in induction at 3 hours after IR. Interestingly, the protein level of Chk2pT68 decreased transiently in both cell lines from the hour 6 to 48, with the largest decrease at hour 24, irrespective of p53 status, indicating oscillating course of DDR in HCT116 cells (Geva-Zatorsky et al., 2006). In p53 wild type cells, serine 15 phosphorylation of p53 possessed similar trend as Chk2pT68.

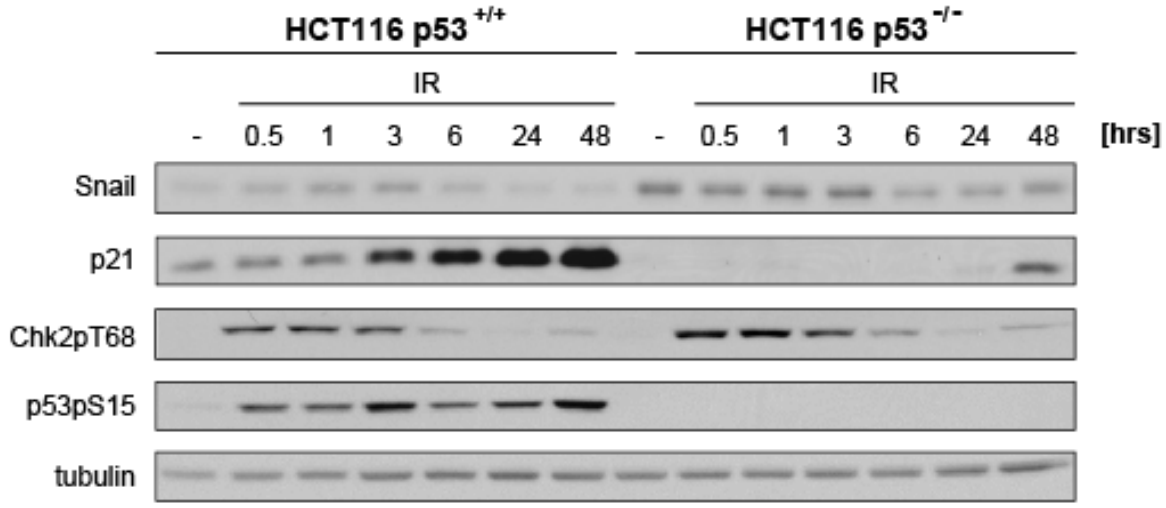


Figure 12. Correlation between Snail and p21 levels in irradiated HCT116 cells. Immunoblotting detection of Snail, p21, Chk2 phosphorylated on threonine 68 (Chk2pT68) and p53 phosphorylated at serine 15 (p53pS15). γ-tubulin was used as a loading control. HCT116 p53 wild-type (HCT116 $p53^{+/+}$) and HCT116 p53 negative (HCT116 $p53^{-/-}$) were irradiated (2 Gy) and harvested 0.5, 1, 3, 6, 24 and 48 h after IR exposure. (L. Kyjacova (unpublished data))

To decipher whether Snail has a direct role in p21 regulation in response to IR, we decided to down-regulate Snail levels by siRNA interference in the same cell lines. As evaluated with real time qRT-PCR, siRNA knock-down of Snail resulted in a decrease of Snail mRNA level in both $p53^{+/+}$ and $p53^{-/-}$ cell lines (Figure 13a). Importantly, knock-down of Snail resulted in increase of p21 mRNA level in both cell types independently of p53 status. However, a clear difference between HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells was observed at the level of p21 mRNA, strictly under DNA damage conditions. The increase of p21 mRNA in HCT116 $p53^{+/+}$

compared to HCT116 p53$^{-/-}$, both after IR exposure, indicating p53-dependent induction of p21 overcharging the suppressive effect of Snail.

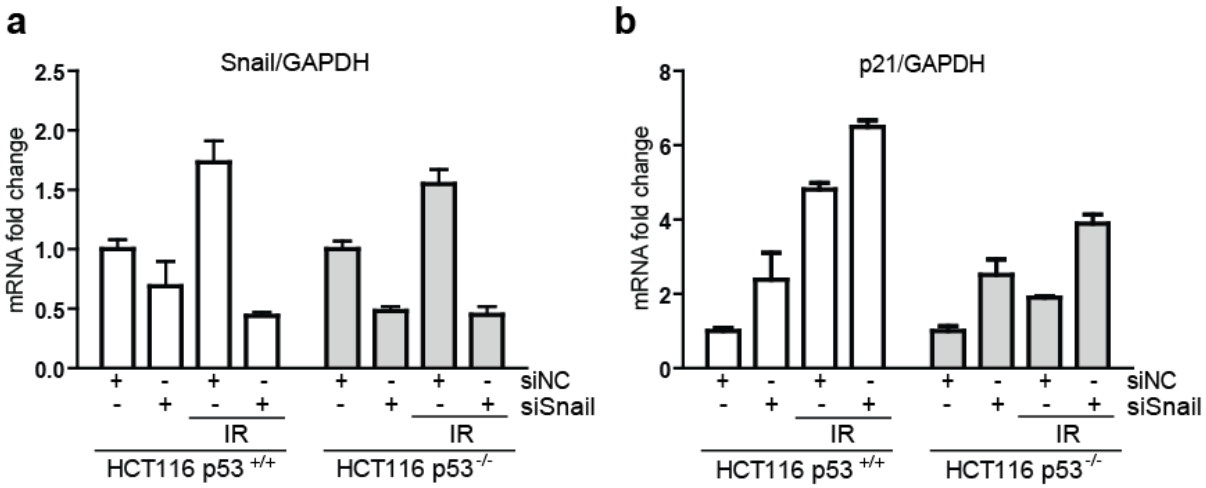
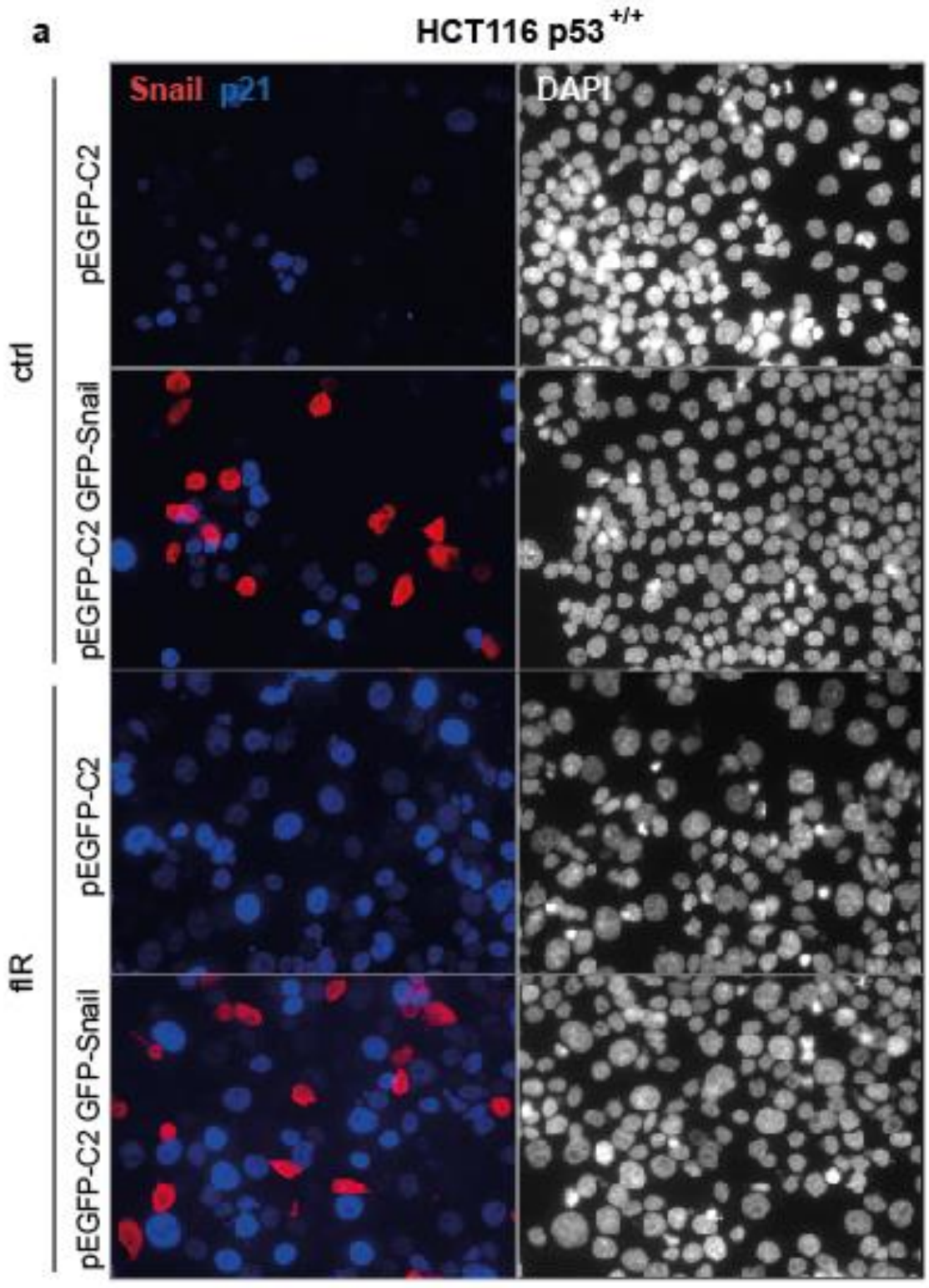


Figure 13. Knock down of Snail results in increased p21 mRNA levels in both HCT116 p53$^{-/-}$ and HCT116 p53$^{+/+}$ cells. Snail (a) and p21 (b) mRNA levels were determined by real-time qRT-PCR. GAPDH was used as a reference gene. HCT116 p53$^{-/-}$ and HCT116 p53$^{+/+}$ cells were transfected twice (with an interval of 48 h) with Snail siRNA (siSnail) or non-targeting siRNA sequences (siNC) as a negative control. Cells were irradiated with 2 doses of 2 Gy 48 h after first transfection and harvested 24 h after the last IR dose. Representative data from 2 independent experiments are shown. Data represent mean ± S.D.

We next transfected both HCT116 p53$^{+/+}$ and p53$^{-/-}$ cells with pEGFP-Snail expression vector to overexpress Snail. Both cell types were then irradiated with two doses of 2 Gy. p21 and Snail protein levels were evaluated using indirect immunofluorescence detection. As shown in Figure 14, Snail (red signal) was present in the cells that did not express p21 (blue signal) and *vice versa*. This effect was again independent on p53 status of HCT116 cells. The increase of Snail positive cells in HCT116 p53$^{-/-}$ line correlated with the decrease of p21 positive cells, indicating again the suppressive role of Snail on p21.


a
HCT116 p53 +/+
Snail p21
DAPI
ctrl
flR
pEGFP-C2
pEGFP-C2 GFP-Snail
pEGFP-C2
pEGFP-C2 GFP-Snail

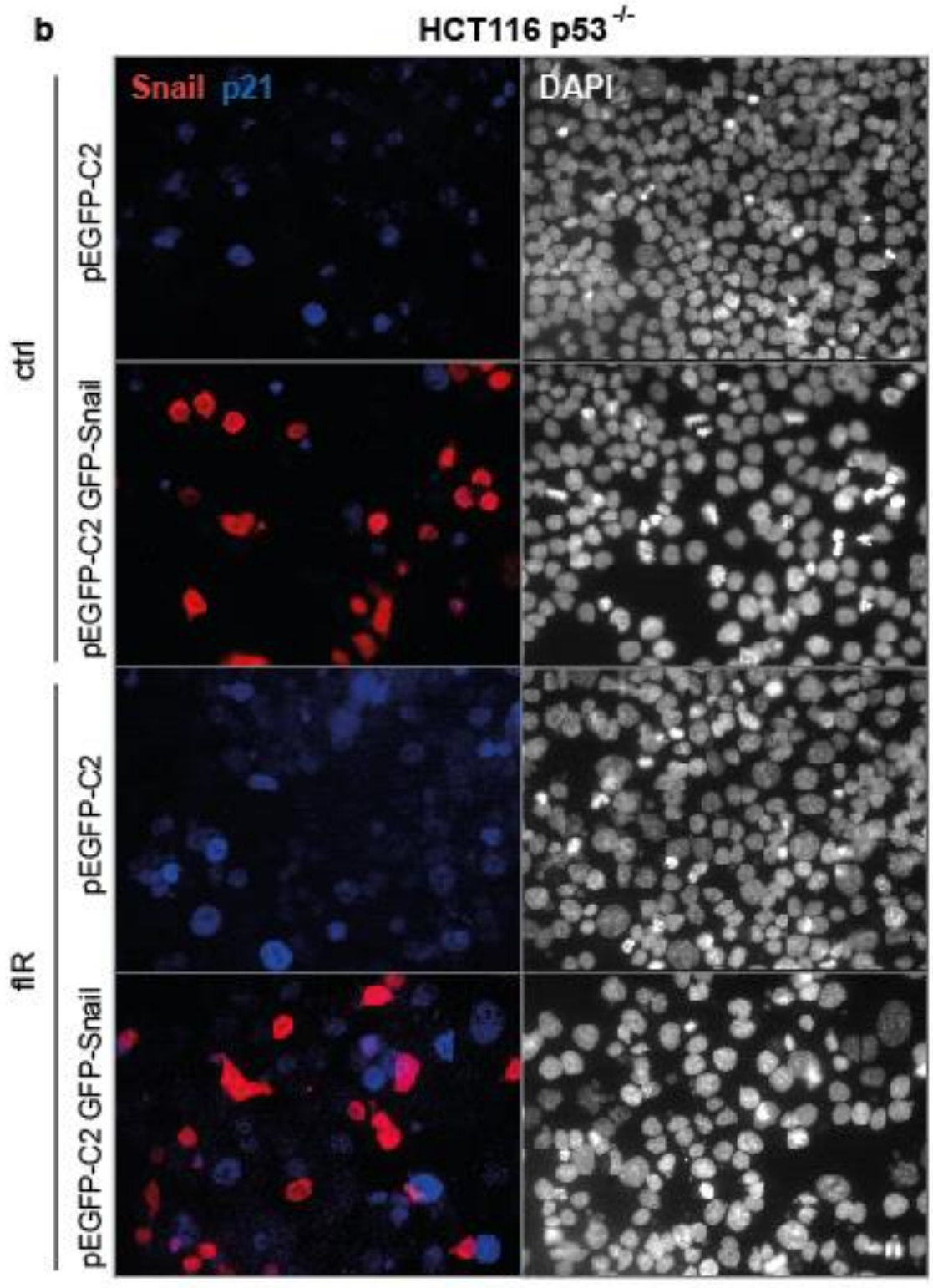


Figure 14. Ectopic expression of Snail negatively correlates with the level of p21 protein independently of p53 status in HCT116 colorectal cancer cell lines. Immunofluorescence detection of p21 and Snail proteins in HCT116 $p53^{+/+}$ (a) and HCT116 $p53^{-/-}$ (b) transfected with either pEGFP-empty or pEGFP-Snail, irradiated (2 x 2 Gy) or not and harvested 24 h after the last IR exposure. DAPI was used to detect cell nuclei (gray). (L. Kyjacova (unpubleshed data))

As described above, PCa cell line DU145 contains mutation in p53 DNA-binding domain, while PC-3 line is p53 null. LNCaP PCa cells possess, in contrast, wild-type p53 gene (Carroll et al., 1993). Thus, we next utilized these three cell lines to analyze the role of Snail in p21 expression in various p53 background. To monitor the potential regulation of p21 by Snail in these cells under the stress condition (IR), we first downregulated Snail protein level by siRNA-mediated knock-down. To reach effective knock-down of Snail, we have had to perform the transfection of siRNA in two consecutive steps. Figure 15 shows a visible decrease of Snail protein levels in Snail siRNA-treated cells, both in irradiated and non-irradiated samples. Indeed, we observed an increase of p21 in cells with Snail knock-down in p53-mutated (DU145) and p53-negative (PC-3) cells both in control and irradiated samples. In LNCaP cells, the induction of p21 following irradiation was significantly higher than in DU145 and PC3 cells, indicating prevailing suppressive role of Snail in cells with aberrant function of p53.

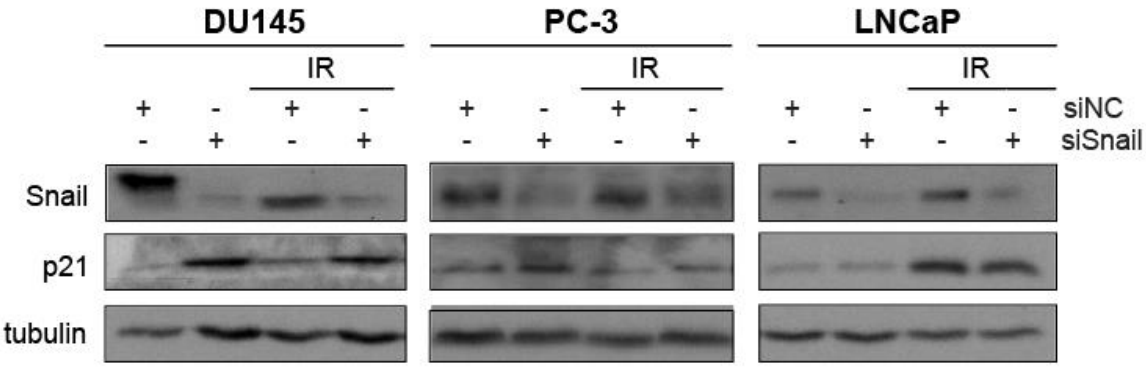


Figure 15. Effect of Snail knock down on fractionated irradiation-mediated p21 induction in PCa cell lines with different p53 status. Immunoblotting detection of Snail and p21 in DU145, PC-3 and LNCaP cells. Cells were transfected twice (with an interval of 48 h between each siRNA transfection) with Snail siRNA (siSnail) or non-targeting siRNA sequences (siNC) used as a negative control. Cells were irradiated with two doses of 2 Gy (interval of 24 h). Cells were harvested 24 h after the last dose of IR. Representative data from 3 independent experiments are shown. γ-tubulin (tubulin) was used as a loading control.

In the next experimental set, mRNA levels of Snail and p21 were detected by real time qRT-PCR after the fIR exposure in all three PCa cell lines in the presence or absence Snail (siSnail), as indicated in Figure 16. Despite using a double transfection of siRNA, we were able to reduce Snail mRNA to desirable levels only in DU145 and LNCaP, but not in PC-3 cells (Figure 16a). Nevertheless, the knock-down of Snail in DU145 cells resulted in significantly increased expression of p21 (Figure 16b). Again, the induction of p21 in LNCaP cells after irradiation was substantially higher than in DU145 and PC3 cells and was not negatively affected by Snail RNA interference, which is in agreement with the data on the protein level (see Figure 15).

Altogether, our data indicate that in case of PCa cells Snail acts as a repressor of p21, and this regulatory role is more pronounced in cells with abrogated function of p53.

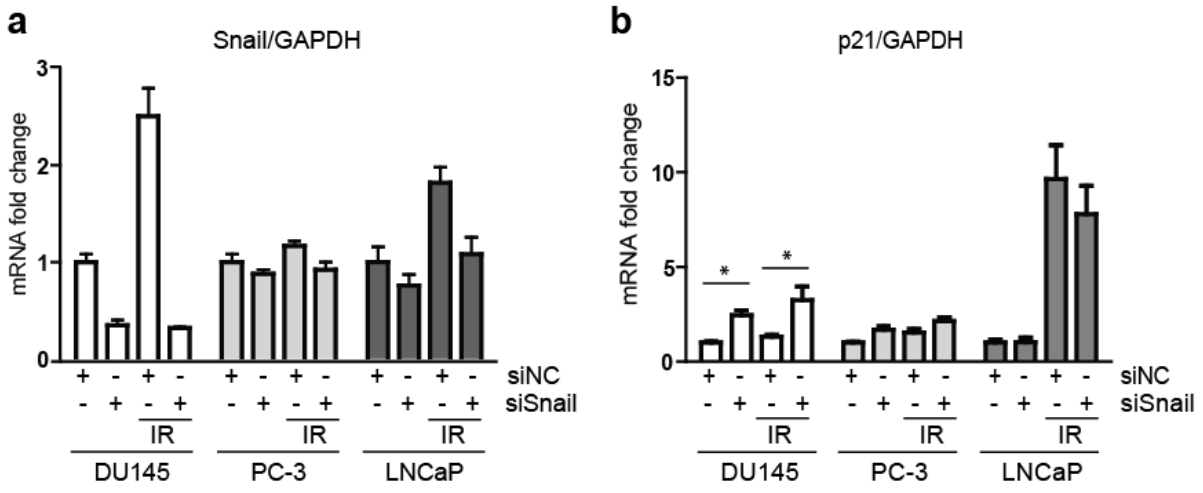


Figure 16 The effect of Snail knock down on stress-induced transcription of p21 in PCa with different p53 status. Snail (a) and p21 (b) mRNA levels were determined by real time qRT-PCR. GAPDH was used as a reference gene. DU145, PC-3 and LNCaP cells were transfected twice (an interval of 48 h) with Snail siRNA (siSnail) or non-targeting siRNA sequences (siNC) as a negative control. Cells were irradiated or not with 2 doses of 2 Gy (interval of 24 h) 48 h after first transfection and harvested 24 h after last IR dose. Representative data from 4 independent experiments are shown. Data represent mean ± S.D. *$p<0.05$.

5.3. Identifying novel transcriptional targets of Snail

Since Snail is well recognized EMT driver in cancer (Cano et al., 2000; Guaita et al., 2002), many EMT-associated genes were identified as its direct transcriptional targets (Cano et al., 2000; Ikenouchi et al., 2003; Takkunen et al., 2006). However, Snail plays a role in many other processes such as apoptosis, DDR or cell cycle regulation (Hu et al., 2008; Kajita et al., 2004; Vega et al., 2004) and the majority of Snail targets have not been identified yet. Therefore, we wished to uncover some more genes that may be regulated by the transcription factor Snail in response to DNA damage.

Importantly, Kurrey *et al.* performed high-throughput chromatin immunoprecipitation and promoter array analysis (ChIP-on-chip) using epithelial ovarian cancer cell line developed to overexpress Snail (A4) (Bapat et al., 2005) and identified 614 genes containing E-boxes representing potential targets for the Snail binding (Kurrey et al., 2009). We used this gene set to try to identify genes involved in DNA repair or cell cycle regulation and regulated by Snail in cells exposed to DNA damaging agents.

In our laboratory, the whole genome gene expression analysis of surviving subpopulations of DU145 irradiated with ten daily doses of 2 Gy was performed. Exposure to fIR in these cells led to the formation of 2 radiation-surviving cell populations – adherent cells with senescence features and non-adherent anoikis-resistant cells with the ability to restore proliferation and adherent growth. Since fIR induced Snail and EMT in PCa cells (Kyjacova et al., 2015), it could be suggested that there is a mechanistic link between EMT and DDR machinery (Boohaker et al., 2013,Zhou et al., 2013,Zhang et al., 2014). Moreover, Snail has been implicated in radioresistance-associated EMT (Escrivà et al., 2008), which is consistent with our data showing the higher levels of Snail in fIR-surviving anoikis resistant non-adherent population.

As the formation and survival of anoikis-resistant non-adherent subpopulation of DU145 and PC-3 cells was dependent on Snail, which level was significantly elevated compared to control cells (Kyjacova et al., 2015), we compared both datasets to find genes that can be regulated by Snail. Identified genes found in both datasets - cyclin B3 (CCNB3) and single strand DNA binding protein 1 (SSBP1, hSSB1), were selected for further analysis to test their regulation by Snail after DNA damage induction (see Figure 17).

CCNB3 is involved in the cell cycle progression, specifically, in transition from G2 phase to mitosis through its association with Cdk2. But, if not properly degraded, it can lead to the cell cycle arrest in G1 and G2 (Tschöp et al., 2006).

hSSB1 is a single-strand binding DNA protein that is essential for efficient repair of DNA double-strand breaks (DSBs). Cells deficient in hSSB1 displayed increased radiosensitivity and defective checkpoint activation, probably through defective amplifying of ATM-dependent signaling (Richard et al., 2008). Furthermore, it was previously observed that hSSB1 can protect p53 and its main downstream target p21 from ubiquitin-mediated degradation (Xu et al., 2011, 2013), resulting in hSSB1 regulation of the cell cycle progression and DNA damage checkpoint.

Figure 17. Snail putative target gene set identified via chip-on-chip (Kurrey et al., 2009) shown as data set no. 2, was compared to genes repressed ($p<0.05$) in DU145 fIR-surviving non-adherent population (see (Kyjacova et al., 2015)) and unpublished data) and selected for involvement in the cell cycle regulation, p53 signaling pathway, base excision repair, nucleotide excision repair, mismatch repair, homologous recombination and non-homologous end-joining (data set no. 1). Genes present in both datasets are marked in red.

To assess the role of Snail in the transcription control of SSB1 and CCNB3, we designed two different experiments. First, we performed siRNA-mediated knock down of Snail in DU145 cells, which were irradiated or not with 2 doses of 2 Gy to induce DNA damage. Levels of Snail, hSSB1 and CCNB3 mRNAs were then determined by real time qRT-PCR (Figure 10a). The effectiveness of siRNA-mediated Snail knock-down was confirmed by measurement the level of Snail mRNA (Figure 18a). Although the level of Snail was decreased, the mRNA levels of hSSB1 and CCNB3 were not influenced both by IR and by Snail knock down (Figure 17a) indicating that their expression is stable and not affected by this type of genotoxic stress.

To underscore these findings, we used alternative approach to ectopically overexpress Snail and test its effect on both genes in cells exposed to genotoxic stress. To this purpose, we transfected MCF-7 and HEK293 cells (easily transfected compared to poorly transfectable PCa cell lines) with pEGFP-Snail (Snail) expression and control pEGFP-empty (empty) vector.

Transfected cells were then treated with the anti-cancer radiomimetic drug neocarzinostatin (NCS). mRNA levels of Snail, hSSB1 and CCNB3 were again determined by real time qRT-PCR. As seen in Figure 18b and 18c, overexpression of Snail had no effect on CCNB3 and hSSB1 mRNA levels both in control and irradiated samples.

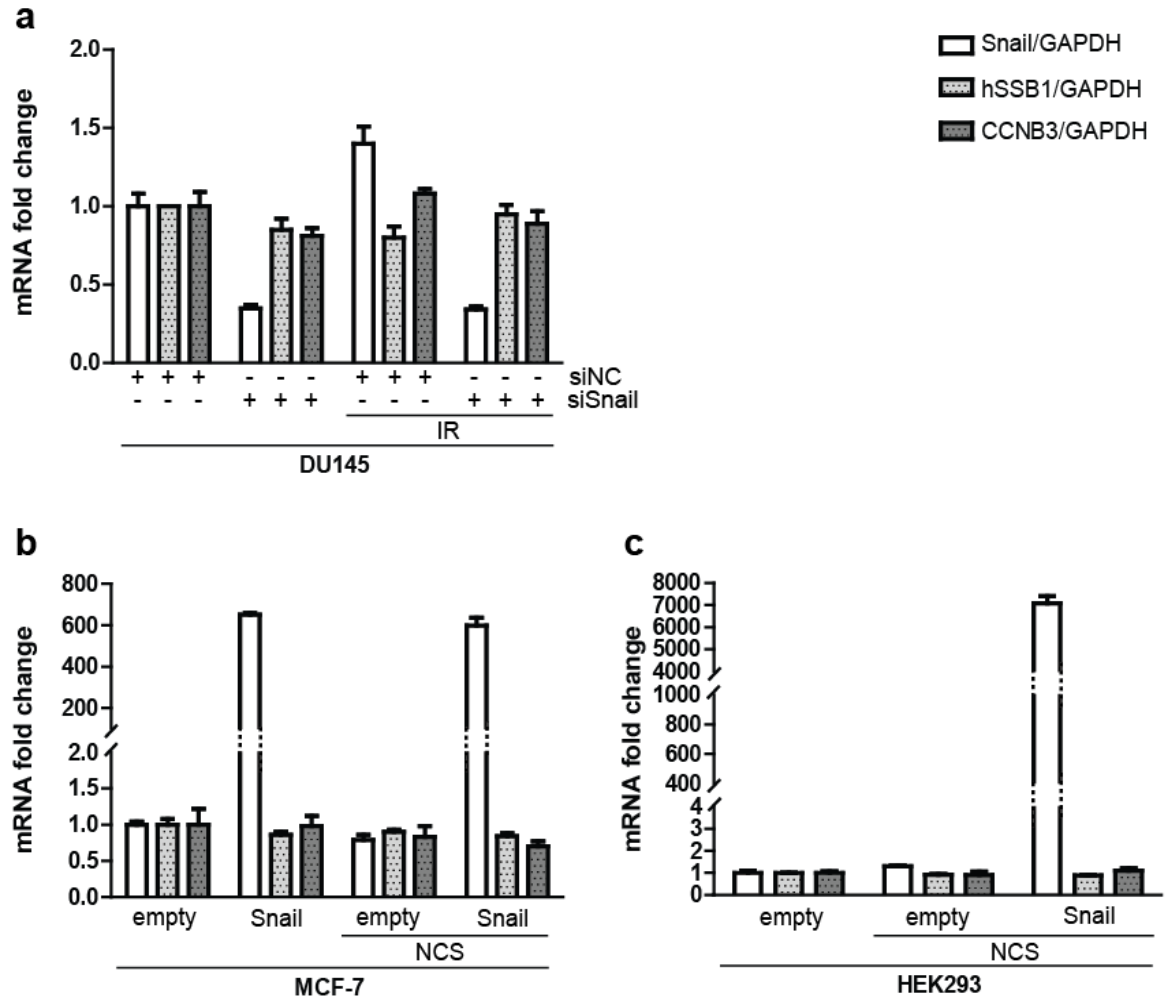


Figure 18. The effect of Snail knock down on mRNA levels of hSSB1 and CCNB3. mRNA levels of Snail, hSSB1 and CCNB3 were determined by real-time qRT-PCR in DU145 exposed to fIR (2 doses of 2 Gy at 24 h interval), and MCF-7 and HEK293 cells treated with radiomimetic drug neocarzinostatine (NCS). GAPDH was used as a reference gene. DU145 cells (a) were transfected twice (an interval of 48 h) with Snail siRNA (siSnail) or non-targeting siRNA sequences (siNC) as a negative control and then irradiated or not with 2 doses of 2 Gy 48 h after first transfection and analyzed 24 h after the last IR dose. MCF-7 (b) and HEK293 (c) cells were transfected with pEGFP-empty (empty) as a negative control or pEGFP-Snail (Snail) vectors, treated or not with neocarzinostatin (NCS, 100ng/ml) and harvested 24 h after treatment. Data were obtained from 2 independent experiments. Data represent mean ± S.D.

Taken together, our findings indicate that Snail does not mediate the transcription of the hSSB1 and of the CCNB3 mRNA in DU145, MCF-7 and HEK293 in both unperturbed conditions and after genotoxic stress.

6. Discussion

6.1. The role of ATM in stabilization of Snail

ATM kinase is a central protein orchestrating the DDR (reviewed by (Shiloh, 2003)). ATM activation upon DNA damage lead to the phosphorylation of hundreds of its target substrates (Matsuoka et al., 2007), which are involved in the regulation of DNA repair, cell cycle, transcription, etc. (reviewed by Medema and Macůrek, 2012). ATM kinase preferentially phosphorylates its substrates on serine or threonine residues that precede glutamine residues, so-called SQ/TQ motifs (Kim et al., 1999).

Unlike other transcription factors, activation and stability of Snail is also driven by posttranslational modifications including phosphorylation. For example, it was demonstrated that GSK-3β-mediated Snail phosphorylation targets Snail to the cytosol for its proteasomal degradation (Zhou et al., 2004). Importantly, Sun and coworkers (Sun et al., 2012) showed that ATM is hyper-activated in breast tumors with lymph-node metastasis and that this hyper-activation correlates with the elevated expression of Snail. Hereafter they found that ATM phosphorylates Snail at serine 100, matching the SQ/TQ consensus motif, the only potential ATM phosphorylation site in the Snail protein sequence (Kastan and Lim, 2000)

They also anticipated that ATM-mediated Snail phosphorylation prevent its GSK-3β-mediated degradation (Sun et al., 2012), as serine 100 is, at the same time, target site for the phosphorylation via GSK-3β (Zhou et al., 2004). Furthermore, it was shown by the same group that this posttranslational modification of Snail regulates tumor cell radiosensitivity and invasion after IR in breast cancer cell lines (Boohaker et al., 2013). As Snail is upregulated in PCa cell lines undergoing anti-cancer genotoxic therapies (Kyjacova et al., 2015; Liu et al., 2015) we decided to investigate whether this effect is also dependent on ATM.

For this purpose, PCa cell line DU145 and breast cancer cell line MCF-7 (used as a positive control), were treated with ATM inhibitor KU55933 and subjected to IR or CPT treatment to induce DNA damage. Although the ATM inhibition led to the decreased Snail levels in both irradiated cancer cells, the resulting decline of Snail levels, particularly in MCF-7 cells, was not as marked as we expected, compared to the results published by Sun *et al*. (Sun et al., 2012). Therefore, our next experiments were designed to repeat exactly the same conditions used in the study of Sun *et al*. (see Figure 9; (Sun et al., 2012)) with MCF-7 (and DU145) cells treated with CPT. Despite the effective inhibition of ATM proved by decreased

phosphorylation of its target Chk2, the ATM-mediated Snail stabilization has not been clearly demonstrated, since the Snail reduction is not apparent in all samples treated with ATM inhibitor and CPT. The same results were achieved in several independent experiments.

Although the reason for such discrepancy is unknown, one should take into account the accumulating differences in cell lines of the same origin due to the long term propagation in laboratories. Moreover, Snail was previously shown to be stabilized also *via* phosphorylation by p21-activated kinase 1 (Pak1) (Yang et al., 2005), which is rapidly stimulated by IR and also plays a role in the DDR (Falck et al., 2001). Thus the inhibition of ATM itself may not be sufficient to influence the stability of Snail and simultaneous inhibition of both kinases (Pak1 and ATM) may be needed.

To definitely confirm or refuse the role of ATM in the Snail stabilization, it is necessary to conduct further experiments, preferably using MCF-7 cells obtained from another source. I would also propose to inhibit the protein translation and degradation machineries with specific inhibitors, and then monitor the effect of ATM on Snail stability. Alternatively, I would repeat previous experiments using the ATM RNA interference as an alternative to the chemical inhibition of ATM.

6.2. Snail dependent regulation of p21 $^{waf1/cip1}$ expression in DU145 cells

One of the main factors participating in diverse cellular responses to DNA damage is a transcription factor p53. Besides its other functions, activation of p53 may trigger cell cycle arrest through the induction of expression of the Cdk inhibitor p21 (El-Deiry et al., 1994). The p53 gene is one of the most frequently mutated genes in human cancer (reviewed in Brosh and Rotter, 2010; Hollstein et al., 1991), nevertheless p21 was shown to be induced even in p53-negative/inactive cancer cells (Loignon et al., 1997). As the p21 gene promoter contains the Snail-binding consensus motif (E-box) (Kurrey et al., 2009), we addressed the question whether p21 could be transcriptionally regulated via Snail under conditions of the dysfunctional p53.

Using siRNA-mediated gene knock-down of Snail, we demonstrated that Snail level negatively affects both p21 mRNA and protein levels in DU145 PCa cells (carrying single point mutation in DNA binding domain of p53) (Isaacs et al., 1991) contrary to LNCaP cells (which are p53 wild-type). This cell-type difference can be explained just by the p53 status, as the

extensive activation of p21 *via* p53-p21 pathway can mask the suppressive role of Snail on the p21 transcription.

To test this we took the advantage of isogenic HCT116 cell lines that we have in variants with wild type and knocked-out p53 gene (Baker et al., 1989). As we showed, Snail knock down caused induction of p21 in both HCT116 $p53^{+/+}$ and $p53^{-/-}$ cell lines exposed to IR, indicating that the status of p53 is not the main denominator of the presence/absence of Snail suppressive effect on p21, especially in the context of HCT116 cell line. Thus the lack of p21 induction after knock down of Snail in LNCaP cell line can be due to other reasons than the masking effect of p53. For example, one possibility is that Snail does not function as the repressor of p21 in LNCaP cells. Another explanation could be that p21 is not primarily induced by p53 in some cell lines after exposure to genotoxic stress. These findings are consistent with the work of Takahashi *et al.*, who observed the inhibition of the p21 expression by Snail in MG63 osteosarcoma cell line (Takahashi et al., 2004), which has been previously shown to contain mutation in the p53 gene (Masuda et al., 1987). Moreover, Takahashi and coworkers showed that Snail ectopic expression in MG63 cells suppressed E2A-mediated p21 expression, because E2A compete with Snail for the same E-boxes (Takahashi et al., 2004). E2A transcription factor belongs to the basic helix-loop-helix family of proteins and play an important role in the cell cycle progression by regulating transcription of the p21 gene (Prabhu et al., 1997). E2A was also shown to be required for full p21 induction upon p53 activation by both genotoxic and non-genotoxic conditions in multiple cancer cell types expressing wild type p53. Interestingly, E2A seems to act downstream of p53 binding to the p21 enhancers (Andrysik et al., 2013). This may suggest the impact of the malfunctional p53 on E2A activity. As Snail and E2A transcription factors were examined to compete with each other for the same binding sequence, but display the opposite role in the p21 transcription (Takahashi et al., 2004), we assume that altered E2A action, caused by p53 depletion/mutation, may lead to changes in Snail binding to p21 regulatory region. Thus, the Snail repressive effect on p21 can prevail in p53 negative/inactive background and in this way influence the effect of DDR on cell cycle progression. On the other hand, in MDCK (derived from the kidney tissue of an adult female cocker spaniel) cells, Snail was previously shown to induce G0/G1 arrest through the increased expression of p21 (Vega et al., 2004), indicating that Snail can act as transcriptional activator of p21 - in dependence on the cell type context.

As Snail was shown to inhibit expression of the p21 induced by E2A (Takahashi et al., 2004), we would like to investigate in future experiments, if Snail reduces p21 levels in DU145 cells in the same E2A outcompeting manner. To determine this, we can, for example, transfect

DU145 cells with the expression plasmid for E2A and Snail and then examine p21 mRNA and protein levels by real time qRT-PCR and immunofluorescence/immunoblotting, respectively. Moreover, we should explore the relationship between p53 status and E2A activity, especially because E2A expression is considerably high in DU145 and PC-3 PCa cell lines (Patel and Chaudhary, 2012), while in LNCaP cells E2A expression is low to negligible (Asirvatham et al., 2007). We can then consider the E2A expression to substitute p53-mediated p21 induction in DU145 and PC-3 cells, which can be subsequently affected by Snail.

6.3. Identifying novel transcriptional targets of Snail

As mentioned above, Snail transcription activity relies on binding to E-boxes of its target genes in order to regulate their transcription (Batlle et al., 2000; Cano et al., 2000). It is well known that Snail is able to induce a complete EMT and it is regulator of plethora factors involved in the basement membrane and extracellular matrix degradation (Zhang et al., 2005). However, its role in the regulation of factors involved in DDR is not clear. Therefore, we compared the whole genome gene expression data of surviving (radioresistant) subpopulations of irradiated DU145 cells, especially significantly repressed genes in non-adherent fraction where the level of Snail was reported high comparing with control cells, (see (Kyjacova et al., 2015)) with genes known to comprise E-boxes on their promoters (Kurrey et al., 2009). In this set of hundreds genes, we identified two genes - hSSB1 and CCNB3 - known to participate in DNA damage response and cell cycle regulation as candidates for Snail-dependent regulation.

hSSB1 belongs to the single-stranded DNA binding protein family and is essential for efficient repair of DNA DSBs by the homologous recombination pathway. hSSB1 is rapidly recruited to the sites of DSBs, interacting with MRN complex (Richard et al., 2011) and this way promotes the ATM activation. Moreover, hSSB1 was shown to protect p53 from degradation and modulate its transcriptional activity (Xu et al., 2013). The similar stabilizing effect of hSSB1 was also observed for p21 (Xu et al., 2011). These data suggest a key role of hSSB1 in DDR.

As the hSSB1 promoter contains the Snail-binding motif, we tested whether Snail has a role in its expression after DNA damage. Knock down of Snail did not significantly affect mRNA levels of hSSB1 in DU145 cells, even after IR treatment. Overexpression of Snail and treatment with NCS in MCF-7 and HEK293 cells did not cause marked changes in hSSB1 mRNA levels, as well. Although these results suggest that there is a no relationship between

Snail and hSSB1expression, it is not clear, why the expression of hSSB1 was not influenced by DNA damage, as reported by Richard *et al.* (Richard et al., 2008). Richard *et al.*, showed that hSSB1 localizes to damaged nuclear foci that have been formed within 30 min of DNA damage and persists up to 8 hours (Richard et al., 2011). This point to the possibility that 24 hours time-point after IR exposure and NCS treatment used by us could be beyond the window of measurable hSSB1 mRNA changes in dependence on Snail, but not definitely means that there is no actual relationship between the two. To answer this question, earlier time-points after IR need to be evaluated.

As the activation of DDR, among others, leads to the cell-cycle arrest and the DDR effectors can directly target the cell-cycle control machinery, cyclins are/can be also considered components of DDR machinery. The representatives of B-type of cyclins that control transition from the G2 phase of cell cycle to mitosis include also CCNB3 (Satyanarayana and Kaldis, 2009).

According to our knowledge, the role of DNA damage on CCNB3 expression has not been investigated yet. To test the role of Snail in transcriptional control of CCNB3, we reduced Snail levels in DU145 using siRNA-mediated gene knock down and subjected cells to IR exposure. Alternatively, MCF-7 and HEK293 cells were transfected with pEGFP-Snail expression vector to overexpress Snail and were treated with NCS to induce DNA damage. Although the whole genome gene expression analysis performed in our laboratory (data not shown) recorded changed expression of CCNB3 in DU145 fIR-surviving non-adherent cells overexpressing Snail, we did not seen changes in CCNB3 mRNA levels induced by treatment with DNA damaging agents. Moreover, no correlation between the Snail levels modulation and the expression of CCNB3 gene was observed. To conclude, our findings indicate that despite cyclin B3 promoter contains putative Snail-binding sequence, Snail is not involved in transcriptional control of CCNB3 gene in DU145, MCF-7 and HEK293 cells.

7. Summary

Optimal DDR is critical for the maintenance of genetic stability and is important in developing radioresistance of cancer cells. However, the role of DDR in tumor progression and metastasis is less understood. Here, we examined and demonstrated the interplay between the transcription factor Snail and DDR. The results of the experimental part of this thesis indicate the following:

1. The stabilization effect of the main DDR effector kinase ATM was not clearly demonstrated, as the ATM inhibition did not caused apparent and reproducible decrease of Snail in DU145 PCa cell line and MCF-7 breast cancer cell line undergoing CPT treatment.

2. Snail can act as the repressor of Cdk inhibitor $p21^{waf1/cip1}$. Its repressor effect is unmasked in cancer cell lines with abrogated function of p53.

3. The role of Snail in the transcription regulation of DDR associated factors hSSB1 and CCNB3, containing putative Snail binding sites (E-boxes) in their promoters, was not confirmed.

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