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DNA damage recognition in the normal epithelium of human prostate and seminal vesicles

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ACADEMIC DISSERTATION

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Abstract

Prostate cancer is currently one of the most prevalent types of cancer in men. The development of prostate tumors has long been known to require exposure to androgens, and several important pathways governing cell growth have been found to be deregulated in prostate tumorigenesis. Recent genetic studies have revealed that complex gene fusions and copy - number alterations are frequent in prostate cancer, which is a unique feature among solid tumors. These chromosomal aberrations are though to arise as a consequence of faulty repair of DNA double strand breaks (DSB). Most repair mechanisms have been studied in detail in cancer cell lines, but how DNA damage is detected and repaired in normal differentiated human cells has not been widely addressed. The events leading to the gene fusions in prostate cancer are under rigorous studies, as they not only shed light on the basic pathobiologic mechanisms but may also produce molecular targets for prostate cancer treatment and prevention.

Prostate and seminal vesicles are part of the male reproductive system. They share similar structure and function but differ dramatically in their cancer incidence. Approximately fifty primary seminal vesicle carcinomas have been reported worldwide. Surprisingly, only little is known on the reasons why seminal vesicles are resistant to neoplastic changes. As both tissues are androgen dependent, it is truly a mystery that androgen signaling would only lead to tumors in prostate tissue.

In this work, we set up novel *ex vivo* – human tissue culture models of prostate and seminal vesicles, and used these to study how DNA damage is recognized and repaired in normal epithelium. Activation of a major DNA - damage inducible pathway, mediated by the ATM kinase, was robustly activated in all main cell types of both tissues. Interestingly, we discovered that secretory epithelial cells had less histone variant H2A.X (H2AX) and after DNA damage much lower levels of H2AX was phosphorylated on serine 139 (γH2AX) than in basal or stromal cells. γH2AX has been considered essential for efficient DSB repair, but as there were no significant differences in the γH2AX levels between the two tissues, it seems more likely that the role of γH2AX is dispensable in postmitotic cells. We also gained insight into the regulation of p53, an important transcription factor that protects genomic integrity via multiple mechanisms, in human tissues. DSBs did not lead to a pronounced activation of p53, but treatments causing transcriptional stress, on the other hand, were able to launch a notable p53 response in both tissue types. In general, *ex vivo* – culturing of human tissues provided unique means to study differentiated cells in their relevant tissue context, and is suited for testing novel therapeutic drugs before clinical trials.

In order to study how prostate and seminal vesicle epithelial cells are able to activate DNA damage – induced cell cycle checkpoints, we used cultures of primary prostate and seminal vesicle epithelial cells. To our knowledge, we are the first to report isolation of

human primary seminal vesicle cells. Surprisingly, human prostate epithelial cells did not activate cell cycle checkpoints after DSBs in part due to low levels of Wee1A, a kinase regulating CDK activity, while primary seminal vesicle epithelial cells possesses proficient cell cycle checkpoints and expressed high levels of Wee1A. Similarly, seminal vesicle cells showed a distinct activation of the p53 - pathway after DSBs that did not occur in prostate epithelial cells. This indicates that p53 protein function is under different control mechanisms in the two cell types, which together with proficient cell cycle checkpoints may be crucial in protecting seminal vesicles from endogenous and exogenous DNA damaging factors and, as a consequence, from carcinogenesis.

These data indicate that two very similar organs of male reproductive system do not respond to DNA damage in a similar manner. The differentiated, non - replicating cells of both tissues were able to recognize DSBs, but under proliferation human prostate epithelial cells had deficient activation of the DNA damage response. This suggests that prostate epithelium is most vulnerable to accumulating genomic aberrations under conditions where it needs to proliferate, for example after inflammatory cellular damage.

Table of Contents

List of original publications

This thesis is based on the following publications:

I **Jäämaa S***, Af Hällström TM*, Sankila A, Rantanen V, Koistinen H, Stenman UH, Zhang Z, Yang Z, De Marzo AM, Taari K, Ruutu M, Andersson LC, Laiho M. DNA damage recognition via activated ATM and p53 pathway in nonproliferating human prostate tissue. *Cancer Res.* 1;70(21):8630-41 (2010)

II Kiviharju - af Hällström TM, **Jäämaa S**, Mönkkönen M, Peltonen K, Andersson LC, Medema RH, Peehl DM, Laiho M. Human prostate epithelium lacks Wee1A - mediated DNA damage - induced checkpoint enforcement. *Proc Natl Acad Sci U S A.* 24;104(17):7211-6 (2007)

III **Jäämaa S**, Sankila A, Rantanen V, Peltonen K, Järvinen PM, af Hällström TM, Ruutu M, Taari K, Andersson LC, Laiho M. Contrasting DNA Damage Checkpoint Responses in Human Cancer Prone Prostate and Cancer Resistant Seminal Vesicle. *The Prostate.* In press (2011)

The publications are referred to in the text by their roman numerals. II has previously been included in the Ph.D. thesis of Taija af Hällström.

* The authors contributed equally to the study.

Abbreviations

5 - BrdU	5 – Bromo - deoxyuridine
53BP1	p53 - binding protein - 1
α - SMA	α - smooth muscle actin
AID	activation - induced cytidine deaminase
AR	androgen receptor
A - T	ataxia telangiectasia syndrome
ATM	ataxia telangiectasia mutated
ATR	ATM and Rad3 related protein kinase
BER	base excision repair
BRCA	breast cancer susceptibility protein
CDC25	cell division cycle 25 phosphatase
CDK	cyclin - dependent kinase
CHK	checkpoint kinase
CIN	chromosomal instability
CK	casein kinase
CPT	camptothecin
CT	computed tomography
DDR	DNA damage response
DHT	dihydrotestosterone
DSB	DNA double strand break
DNA - PK	DNA - dependent protein kinase
DNA - PKcs	DNA - dependent protein kinase, catalytic subunit
dsDNA	double - stranded DNA
DUB	deubiquitinating enzyme
ERG	v - ets erythroblastosis virus E26 oncogene homolog
ES	embryonic stem cell
ETS	E - twenty six transcription factor family
ETV	ETS variant gene
EZHA	enhancer of zeste homolog 2
FACS	fluorescence - activated cell sorting
H2AX	histone variant H2A.X
H2B	histone 2B
H3 - K9me	H3 methylated on lysine 9
H3 - K79me	histone H3 methylated on lysine 79
H4 - K20me2	histone H4 dimethylated on lysine 20
γH2AX	Ser139 phosphorylated histone variant H2A.X
HAUSP	herpesvirus - associated ubiquitin-specific protease
HMEC	human mammary epithelial cell
HP1 - β	heterochromatin protein 1 - β
HPEC	human prostate epithelial cell
HR	homologous recombination
HSVEC	human seminal vesicle epithelial cell
IR	ionizing radiation
IHC	immunohistochemistry

JNK	c - Jun N - terminal kinase
KAP - 1	KRAB - associated protein, also known as TIF1β, KRIP - 1 or TRIM28
MAPK	mitogen - activated protein kinase
MDC1	mediator of DNA - damage checkpoint 1
Mdm2	murine double minute - 2
Mdm4/x	Mdm2 related protein
MEF	mouse embryo fibroblasts
MSI	microsatellite instability
Mre11	meiotic recombination 11
MRN	Mre11 - Rad50 - Nbs1 -complex
mTOR	mammalian target of rapamycin
Nbs1	Nijmegen breakage syndrome protein 1
NER	nucleotide excision repair
NHEJ	non - homologous end – joining
OIS	oncogene - induced senescence
PAP	prostate acid phosphatase
PARP	poly (ADP-ribose) polymerase 1
PIK3CA	phosphoinositide – 3 - kinase, catalytic, alpha polypeptide
PIKK	phosphatidylinositol -3 kinase - related kinase
PIN	prostate intraepithelial neoplasia
Plk1	polo - like kinase 1
PSA	prostate specific antigen
PTEN	phosphatase and tensin homologue
RB	retinoblastoma protein
RDS	radioresistant DNA synthesis
SCID	severe combined immunodeficiency
SMG - 1	suppressor of morphogenesis in genitalia
SVCA	primary seminal vesicle carcinoma
TMPRSS2	transmembrane serine protease isoform 2
TOP1	topoisomerase I
TOP2	topoisomerase II
TP53	tumor protein p53 gene
TRAMP	transgenic adenocarcinoma of mouse prostate
TRRAP	transformation / transcription domain - associated protein

Introduction

Prostate cancer causes significant morbidity and mortality in the western countries. It affects more than 4000 men in Finland every year, and poses diagnostic and therapeutic challenges. Although the molecular events leading to prostate neoplasms are beginning to emerge, we still do not know why prostate cancer is so common. Recent research has indicated that prostate adenocarcinomas harbor a high number of chromosomal aberrations that are uncommon in other solid tumors. First fusion between androgen regulated transmembrane serine protease isoform 2 (TMPRSS2) gene and a transcription factor E - twenty six transcription factor family (ETS) - family member v - ets erythroblastosis virus E26 oncogene homolog (ERG) was described 2005, and since then other gene fusions have been reported. The fusion genes deregulate many important pathways controlling cell growth and proliferation, one the most well - known among them being androgen signaling pathway. The exact mechanisms that lead to accumulation of gene fusions in prostate cancer are currently unknown, but they are though to arise from misrepaired DNA double strand breaks (DSBs). One of the repair pathways important in DSB repair, non - homologous end joining (NHEJ), has been implicated. Therefore, thorough understanding of DNA damage recognition and repair in normal prostate epithelium helps shed light on early events in prostate tumorigenesis.

Seminal vesicles are a part of the male reproductive system and are located at the base of the prostate. Prostate and seminal vesicles are both androgen dependent and share a similar structure and function. Remarkably, primary seminal vesicle carcinomas are extremely rare, as only some 50 cases have been reported worldwide. Therefore, there must be fundamental differences in either the nature and amount of DNA damage, or the way the two tissues are able to recognize and repair DNA damage. Together prostate and seminal vesicles form an ideal model to study the consequences of DNA damage in slowly renewing human epithelium.

Review of the literature

1. DNA damage, genomic instability and prostate cancer

1.1. DNA damage as a cause of cancer

Cancer arises as a result of accumulating mutations in the genetic material of eukaryotic cells (Stratton *et al.*, 2009). The structure of double - stranded DNA (dsDNA) is inherently rather stable, and multiple pathways have evolved to monitor and maintain the integrity of the genome (Hoeijmakers, 2001). Nonetheless, endogenous and exogenous stress factors constantly challenge the stability of the DNA and occasionally genetic lesions escape the correct repair mechanisms. Over time more and more genetic aberrations are fixed into the genomes of our cells and gateway to cancer is opened (Stratton *et al.*, 2009).

There are several distinct classes of somatic mutations that are created by different mechanisms: changes of a single base to another, insertions or deletions of DNA segments, changes in gene copy number or chromosomal rearrangements where two pieces of DNA are falsely joined together. Epigenetic changes do not change the DNA sequence itself but alter the transcription of genes and can thus affect the phenotype of a cancer cell. Completely new DNA sequences may be introduced into DNA by tumor viruses. The rate and type of somatic mutations depends on many factors, including the nature of genotoxic stress and cell type. For example, proliferating epithelial cells of the colon are much more likely to accumulate mutations due to DNA polymerase errors than quiescent neurons. Not all somatic mutations contribute to tumorigenesis, as most of them do not give cells any clonal growth advantage. This type of changes are called passenger mutations, as they are propagated in cancer cells passively by the rare number of mutations that are required to deregulate normal growth programs. It has been estimated that around five - seven genetic alterations are needed to convert normal cells into cancer cells (Schinzel and Hahn, 2008). As these changes drive the tumor formation, they are called the driver mutations.

In the updated version of the most cited review article on cancer Hanahan and Weinberg list genomic instability and mutation as an enabling characteristic that allows the acquisition of cancer cell hallmark properties (Hanahan and Weinberg, 2011). Others have proposed that genomic instability is such an essential feature of cancer cells, that is warrants it to be called as a hallmark of cancer (Negrini *et al.*, 2010). There are different forms of genomic instability. Chromosomal instability (CIN) can be further divided into changes of chromosome structure or number. Microsatellite instability (MSI) changes the number of oligonucleotide repeats in microsatellite regions, and increases the frequency of base - pair mutations. Genomic instability in hereditary cancers is often due to inactivation

of the caretaker genes, e.g. ataxia – telangiectasia mutated (*ATM*) in ataxia telangiectasia (A - T) or BRCA1 or BRCA2 in hereditary breast cancer, but the source of genomic instability in non - hereditary cancer cases is still unclear (Negrini *et al.*, 2010). High - throughput sequencing studies suggest that mutations in DNA repair genes are infrequent and that it is rather oncogene-induced replicative stress that acts a source of genomic instability in sporadic human tumors (Negrini *et al.*, 2010).

1.1.1. Mechanisms protecting cells from tumorigenesis

Hanahan and Weinberg (2011) list three pathways that are important in maintaining genomic integrity: p53, "the guardian on the genome", that is central in monitoring DNA damage and orchestrating a proper cellular response after such events; caretakers of the genome that directly detect and repair damaged DNA or inactivate DNA damaging agents; and telomerase enzyme that protects telomeric DNA and thus guards karyotypic stability. These molecules are indeed essential for preventing multistep tumor progression. Maintenance of genomic integrity in multicellular organisms requires the concerted action of many partially overlapping pathways that control DNA repair, cell cycle progression, fidelity of DNA replication and segregation, protection of chromosomal ends, senescence and apoptosis (Harper and Elledge, 2007; Rouse and Jackson, 2002 and **Figure 1.**). Collectively, all the responses designed to minimize the impact of DNA damage via promotion of DNA repair and cell cycle checkpoints are referred to as DNA damage response (DDR) (Ciccia and Elledge, 2010; Jackson and Bartek, 2009).

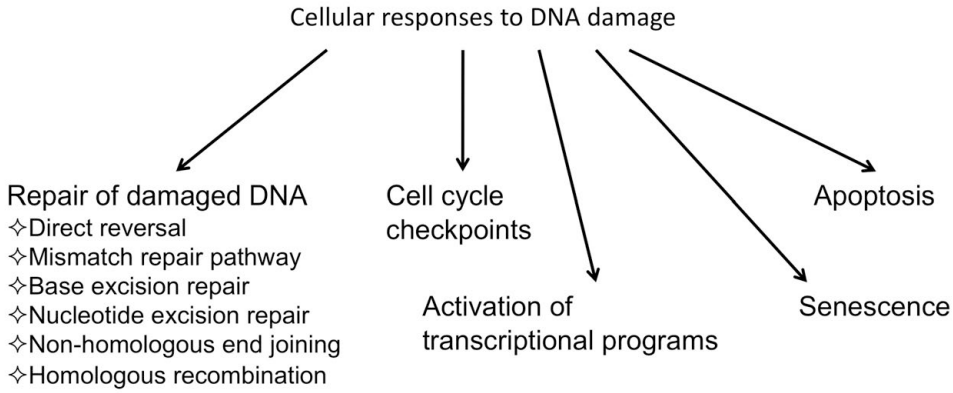


Figure 1. *DNA damage activates various signaling pathways aimed at maintenance of genomic integrity.*

Eukaryotic cells have several different DNA damage repair pathways. Each pathway is responsible for the repair of only certain types of DNA damage (Hakem, 2008). Sometimes it is possible to directly reverse the DNA damage. For example, the enzyme mammalian O6 – methylguanine - DNA methyltransferase (MGMT) is able to remove alkyl groups from

damaged bases without further ado. For more complex lesions, more complex repair pathways are required. Mismatch repair pathway (MMR) detects and repairs small insertions and deletions that occur during DNA replication. It seems to be important especially in the gastrointestinal tract. Failure of this pathway commonly results in MSI. Nucleotide excision repair (NER) is required for removal of lesions that distort the DNA double helix, such as bulky DNA adducts (Cleaver, 2005), whereas base excision repair (BER) is able to replace damaged bases with new nucleotides. For the repair of DSBs, the most lethal form of DNA damage, there are two main pathways called homologous recombination (HR) and NHEJ (Sarasin and Stary, 2007; Sonoda *et al.*, 2006). HR is said to be error - free, as it is able to use the sister chromatid as a template for the repair. On the other hand, this limits HR only to certain phases of cell cycle, when homologous chromosomes are available (Branzei and Foiani, 2008). NHEJ is the predominant pathway for DSB repair in mammalian cells. It is able to ligate broken DNA ends together in all the cell cycle phases, especially in G1, but is unable to prevent DNA base loss in the process and is thus called error - prone. The core components of NHEJ are Ku70 and Ku80 proteins that recognize broken DNA ends, DNA-dependent protein kinase, catalytic subunit (DNA - PKcs) that complexes with Ku proteins to form the functionally active DNA - PK complex that phosphorylates other DNA repair targets and is together with Artemis involved in end processing, and XRCC4 and DNA ligase IV (LigIV) that perform the final ligation of the damaged DNA ends (Mahaney *et al.*, 2009).

Cell cycle regulation constantly monitors mitogenic and anti - mitogenic signaling and thus prevents unscheduled proliferation. There are four phases in the cell cycle: G1, S, G2 and M. Cells replicate their DNA during S - phase, in M - phase they divide into two daughter cells, and the gap phases in between are called G1 and G2. Cyclins and cyclin – dependent kinases (CDKs) form the engine that drives the cell cycle, and CDK activity is further modulated by CDK - inhibitors of Ink4, Cip and Kip – families. Proper progression through these phases is monitored by several cell cycle checkpoints that verify successful completion of each phase of cell cycle before progression into the next one and sense possible defects in DNA synthesis and chromosome segregation. This machinery is commonly deregulated in human cancer (Malumbres and Barbacid, 2009). DNA damage is often said to activate cell cycle checkpoints, although a more proper term, when talking about the signaling cascades that arrest cell cycle progression until DNA damage is repaired, would be ‘DNA damage checkpoints’. Nonetheless, activation of both of these checkpoints leads to inhibition of CDK - activity (Cerqueira *et al.*, 2009), which justifies the overlapping use of the term cell cycle checkpoints. The checkpoints have an important role in tumorigenesis, as their defects can lead to unscheduled proliferation, increased number of mutations and changes in chromosome structure and number (Kastan and Bartek, 2004; Malumbres and Barbacid, 2009).

In the face of extensive DNA damage, which cannot be repaired, the cells can be permanently removed via apoptosis, or may be directed into senescence. The choice

between senescence and apoptosis is influenced by the cell type and nature of the damage. Cellular senescence is a state of permanent inability to proliferate. It is caused by severe stress, and thus differs from quiescence and terminal differentiation (d'Adda di Fagagna, 2008). It is thought to form a barrier against tumorigenesis, as senescent cells have been detected in premalignant lesions and maintenance of the senescent phenotype has been linked to tumor suppressors INK4A, ARF and p53 (Collado and Serrano, 2010). There are several forms of senescence distinguished by the inducing event: telomere shortening of normal cells leads to replicative senescence, overexpression of oncogenes results in oncogene-induced senescence (OIS), and senescence triggered by excessive DNA - damage. Common to all these different forms of senescence is that they are thought to depend on activation of DDR. For example, oncogene activation has been shown to lead to DNA replication stress, DSBs and activation of DDR, although there are some claims that this is not a universal requirement for OIS (Efeyan *et al.*, 2009). If the DDR is inhibited, the senescence barrier is lost and tumors become larger and more invasive (Bartkova *et al.*, 2006). The interplay between DDR and senescence may even be more complex. It has been suggested that tumor cells that have overcome the senescence barrier exploit senescence induced heterochromatin changes to dampen further DDR and apoptosis (Di Micco *et al.*, 2011).

1.2. Prostate carcinogenesis

1.2.1. Prostate tissue structure and function

Prostate is the largest accessory gland of the male reproductive system. It is located at the base of the urinary bladder and surrounds prostatic urethra (Moore and Dalley, 2006, illustrated in **Figure 2**.) and consists of glandular structures and a surrounding fibromuscular stroma. Its main function is to secrete fluid rich in nutrients that support semen, and together with seminal vesicles prostate is responsible for producing the bulk of the seminal fluid. Prostate gland can be divided into several anatomically distinct zones, namely peripheral, central, transition and periurethral zones, which differ in their disease profiles (McNeal, 1981). Benign prostatic hyperplasia mostly affects the periurethral region, while approximately 70 % of prostate cancer develops in the peripheral and around 25 % in the transition zone (McNeal, 1981; McNeal *et al.*, 1988). The zones differ slightly in their morphology. Ducts in the peripheral zone are relatively small and surrounded loosely by smooth muscle fibers, while the central zone is characterised by large acini separated by closely - packed smooth muscle fibers (Laczko *et al.*, 2005).

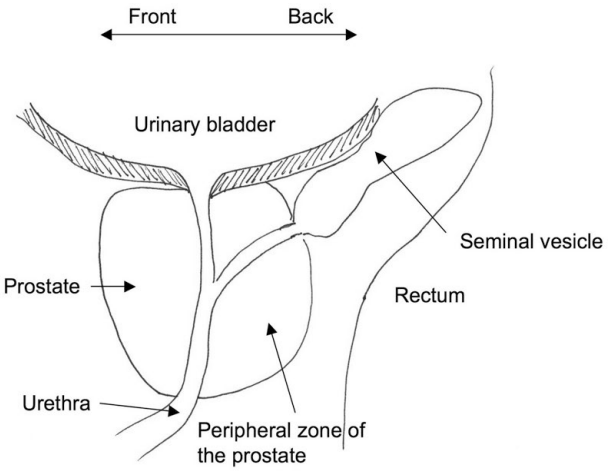


Figure 2. *Schematic illustration of the anatomy of human prostate and seminal vesicles, sagittal view.*

The prostate epithelium consist of two major compartments and five related cell types: basal, luminal and neuroendocrine cells and transit amplifying and stem cells, all of which can be identified by specific markers (Peehl, 2005) and **Figure 3A**. Basal cell compartment consists of small, flattened basal cells that express cytokeratins 5 and 14, very low levels of androgen receptor (AR) and do not express prostate specific antigen (PSA) (Garraway *et al.*, 2003). They can also be identified by expression of p63 (Barbieri and Pietenpol, 2006; Di Como *et al.*, 2002). The exact function of basal cells is unknown, but they are replication - proficient and are thought to be able to differentiate into luminal cells. Luminal compartment consists of terminally differentiated columnar luminal cells that line the glands and are responsible for the secretory functions of prostate. The ratio of basal to luminal cells in prostate is approximately 1:3 (Laczko *et al.*, 2005). The luminal cells express high levels of AR, PSA, prostate – specific acid phosphatase (PAP) and cytokeratins 8 and 18 and are androgen - dependent for survival (Garraway *et al.*, 2003). More than 85 % of them are also positive for cell cycle inhibitor protein p27 (De Marzo *et al.*, 1998). Neuroendocrine cells are rare, constituting 1 - 2 % of prostate cells, and their function is unknown. They are scattered around in the epithelium and can be detected by their expression of chromogranin A or neuron - specific enolase (Laczko *et al.*, 2005; Peehl, 2005). Transit amplifying cells have mostly been characterized in primary cell cultures of prostate epithelium (Garraway *et al.*, 2003; Peehl, 2005; Uzgare *et al.*, 2004). They are thought to represent an intermediate cell type that is not entirely basal or luminal cell, but still more differentiated than prostate stem cell. Transit amplifying cells express both basal and luminal type cytokeratins and have a limited proliferative capacity. Prostate stem cells are androgen – independent, have limitless self - renewal capacity and able to reconstitute all the other epithelial cell types (De Marzo *et al.*, 2010).

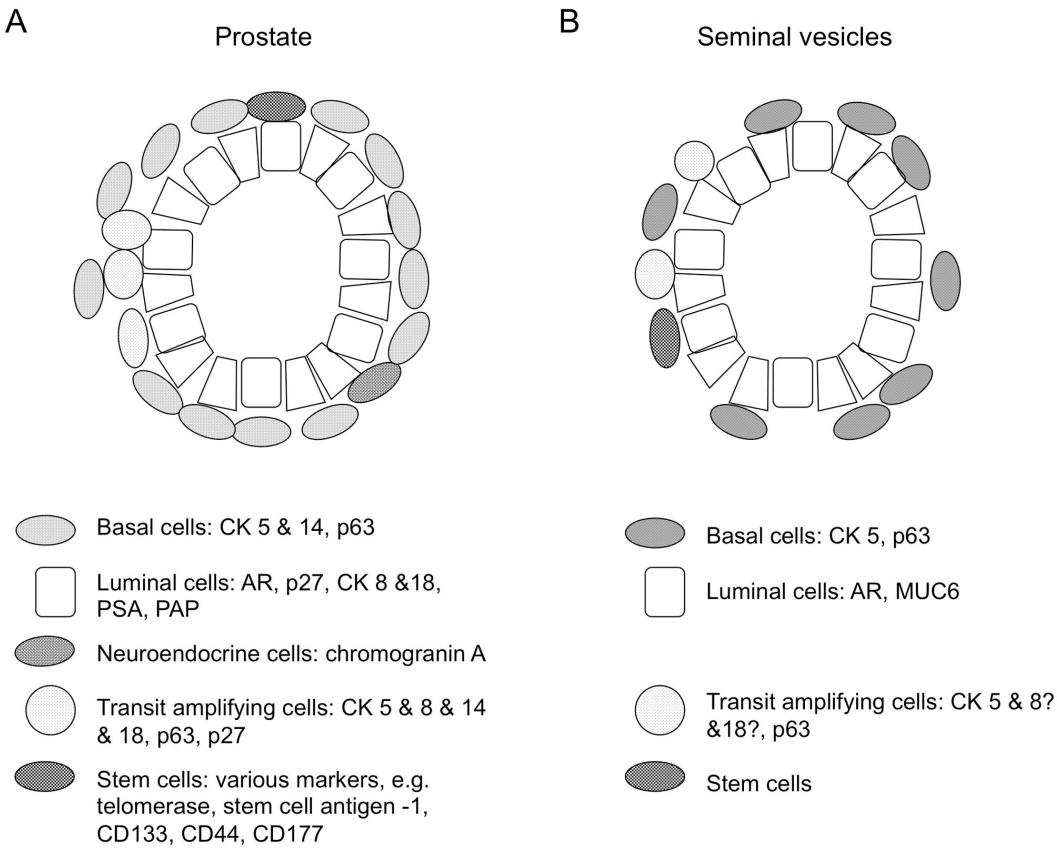


Figure 3. *Prostate (A) and seminal vesicle (B) epithelial structure and cell types. See text for details.*

1.2.2. Prostate carcinogenesis

Prostate cancer is one of the most frequent types of cancer in men with around 4500 new cases diagnosed in Finland and more than 200 000 in USA every year. Prostate cancer incidence has risen steadily, most likely due to enhanced screening and lifestyle changes. Several risk factors for prostate cancer have been described. The most significant ones include aging, family history and ethnic background as well as exposure to androgens (Abate - Shen and Shen, 2000; Shen and Abate - Shen, 2010). It has been suggested that prostate cancer is driven by inflammation caused by viral and bacterial infections, hormonal changes, dietary carcinogens, urine reflux and physical trauma (De Marzo *et al.*, 2007).

Pathological, epidemiological and cytogenic evidence suggest that prostate cancer develops progressively from early low - grade prostatic intraepithelial neoplasia (PIN) – lesions through high grade PIN into full - blown cancer (Joshua *et al.*, 2008), and in 75 % of cases it is multifocal (Ruijter *et al.*, 1996). The natural course of the disease is usually slow, and refraining from radical treatment options is justified. However, in a significant

number of patients (ca. 15 – 20 % of cases according to Finnish Cancer Registry), prostate cancer is more aggressive, even fatal. A considerable amount of evidence suggests that multiple anatomically separate primary tumors arise independently (Bostwick *et al.*, 1998; Cheng *et al.*, 1998; Macintosh *et al.*, 1998; Mehra *et al.*, 2007; Ruijter *et al.*, 1999), but most lethal metastases of prostate cancer are derived from the same primary lesion and are of monoclonal origin (Liu *et al.*, 2009). Despite years of efforts in finding specific markers for the aggressive, metastatic disease form (Kumar - Sinha and Chinnaiyan, 2003; Seligson *et al.*, 2005; Varambally *et al.*, 2005), it is still challenging to differentiate the patients that do benefit from aggressive treatment.

Most prostate cancer cases are adenocarcinomas of epithelial origin, but which epithelial cell type is the cell of origin for prostate tumors has not been established conclusively. There is evidence to support that targeting of both basal or luminal cells can give rise to prostate tumors (De Marzo *et al.*, 2010; Moscatelli and Wilson, 2010). Luminal cells have been likely candidates as a cell - of origin, as prostate tumor cells have morphological and biochemical similarities to luminal cells and the basal cell layer is missing in prostate tumors. This was recently supported by Wang *et al.* (2009), who showed that deletion of phosphatase and tensin homologue (PTEN) in castration resistant luminal stem cells leads to overexpression of phosphorylated AKT and to a rapid formation of high - grade PIN and carcinomas in mice. There is some evidence that a similar luminal stem cell exist in human prostate (Zhao *et al.*, 2010), but its role in human tumorigenesis has not been addressed. On the other hand, transformation of basal cells could also lead to prostate tumorigenesis. First, isolation of primary epithelial cells has shown that basal cells have better proliferative capacity in cell culture (Huper and Marks, 2007; Peehl, 2005). Second, paracrine stimulation of FGF signaling, ERG overexpression and AKT activation resulted in PIN and prostate cancer in murine basal cell transplanted *in vivo* with urogenital sinus mesenchyme, while luminal cells failed to respond (Lawson *et al.*, 2010). Another group showed that benign primary human prostate epithelial cells expressing basal cell markers keratin 5 and p63 could reconstitute prostate glandular structure when transplanted subcutaneously into immunodeficient mice with murine urogenital sinus mesenchyme and Matrigel, while keratin 18 positive luminal cells could not. More importantly, only basal cells were able to form PIN - lesions and adenocarcinomas after induced AKT, ERG and AR expression in this model, indicating that prostate tumors can develop from basal cells (Goldstein *et al.*, 2010).

1.2.3. Genetic changes in prostate tumors

Characterization of genetic and epigenetic changes in prostate cancer cells has produced many important advances in understanding the steps leading to tumorigenesis. As prostate tumors are quite heterogeneous, it is likely that there are several combinations of genetic

events that can lead to prostate cancer, but some common themes have been described (Joshua *et al.*, 2008; Taichman *et al.*, 2007). Early events that lead to disturbed regulation of proliferation and decreased apoptosis include downregulation of CDKN1B expression (encodes the cell cycle inhibitor p27), loss of PTEN (activates AKT – pathway), deregulation of the TGF - beta pathway and loss of NKX3.1. These are followed by loss of GSTP1, an enzyme that reduces carcinogenic burden (Hayes *et al.*, 2005), activation of MYC and telomerase enzyme and loss of retinoblastoma (RB), which affects cell cycle progression. At metastatic prostate cancer stage, p53 is lost in 50 % of tumors, and androgen signaling pathways are further disturbed by AR amplification or mutations (Reviewed in De Marzo *et al.*, 2007; Joshua *et al.*, 2008; Shen and Abate - Shen, 2010; Taichman *et al.*, 2007).

Chromosomal rearrangements are known to occur in leukemias, lymphomas and sarcomas (Nambiar and Raghavan, 2011). There are two main types of rearrangements: either a proto - oncogene and a regulatory region of another gene are fused together, which leads to altered gene expression or fusion of two genes produces a novel fusion protein. These fusions are thought to arise a consequence of faulty repair of DSBs, and were thought to be infrequent in solid tumors. In 2005, a recurrent gene fusion was identified between androgen regulated TMPRSS2 gene and a family of ETS - transcription factors (ERG, ETS variant gene 1 (ETV1), ETS variant gene 4 (ETV4)) (Tomlins *et al.*, 2005). TMPRSS2-ERG was shown to be one of the most common gene fusions detected in solid tumors, found in approximately 50 % of prostate cancer (Kumar - Sinha *et al.*, 2008). The role of ERG overexpression in regulating prostate tumorigenesis has been extensively studied. In mice ERG - overexpression is sufficient to induce PIN - type of changes, but it humans it seems to contribute to the transition from PIN lesions to prostate cancer (Carver *et al.*, 2009; Klezovitch *et al.*, 2008; Tomlins *et al.*, 2008; Zong *et al.*, 2009). ERG overexpression has been reported to disrupt AR signaling and activate a H3K27 methyltransferase enhancer of zeste homolog 2 (EZH2) (Yu *et al.*, 2010). Both these changes abrogate lineage - specific differentiation of prostate cell and promote oncogenic, stem cell like properties (Yu *et al.*, 2010).

The range of genomic alterations in prostate cancer has become more complete by recent reports using advanced technologies. Extensive, integrated analysis of 218 primary and metastatic prostate tumors by focused exon sequencing, copy-number alteration and mRNA expression studies revealed new potential oncogenes (NCOA2) and tumor suppressors (FOXP1, RYBP and SHQ1) and indicated that copy-number alterations define clusters of low- and high-risk disease (Taylor *et al.*, 2010). It also revealed that although single mutations in known oncogenes and tumor suppressor genes (e.g. *PIK3CA, KRAS, BRAF* and *TP53*) are rare, RB signaling pathway driving G1/S progression as well as PI3K and RAS/RAF signaling pathways are altered in a significant number of primary and metastatic prostate tumors (Taylor *et al.*, 2010). In order to characterize the full spectrum of somatic alterations in prostate cancer, Berger *et al.* (2011) sequenced the complete genome of seven

prostate tumors. They discovered that overall mutation rate in prostate cancer was similar to acute myeloid leukemia and breast cancer, but 7 - 15 fold lower than in small cell lung cancer or melanoma (Berger *et al.*, 2011). The rate of genomic rearrangements with median of 90 alterations per genome was similar to what has been described in breast cancer. Interestingly, they also described a novel pattern of complex chain of balanced translocations (illustrated in **Figure 4.**), and suggested that these might arise from erroneous repair of DSBs of genes migrated into same transcription factories or located in the same chromatin compartment. Formation of these inter- and intrachromosomal fusions of multiple genes would deregulate multiple pathways at once and thus efficiently drive tumorigenesis (Berger *et al.*, 2011).

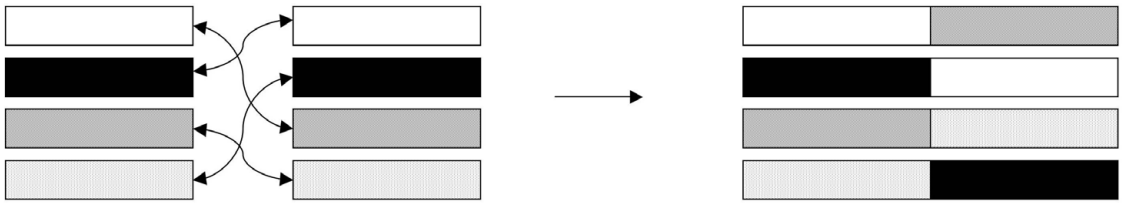


Figure 4. *Complex fusions detected in prostate cancer. Balanced translocations are formed between four inter - and intrachromosomal loci. Modified from Berger et al., 2011.*

1.2.4. How chromosomal translocations are formed in prostate

The importance of chromosomal translocations in prostate tumorigenesis is recognized, and there are some clues on how the fusions, especially between TMPRSS2 - ERG arise. In 1996, Kuettel *et al.* (1996) published that SV40 T – immortalized human fetal prostate epithelial cells can be transformed and formed tumors in athymic mice after a cumulative dose of 30 Gy was reached by repeated exposure to ionizing radiation (IR). The tumors did not harbor point mutations in p53 or ras, but showed numerous chromosomal defects and especially chromosome 3 and 8 translocations (Kuettel *et al.*, 1996). The undisputed role of androgen exposure in formation of neoplasms of prostate, the fact that TMPRSS2 and ERG genes are located 3 Mb apart on the same chromosome and a report indicating that estrogen induced rapid chromosomal movements inspired several groups to investigate whether AR signaling would contribute to gene rearrangements in prostate cancer. First, dihydrotestosterone (DHT) treatment of LNCaP prostate cancer cells was shown to induce proximity between TMPRSS2 and ERG loci in an AR - dependent manner, and a subsequent treatment of the cells with IR lead to the appearance of the fusion in clonally expanded cells (Mani *et al.*, 2009). Second, DHT and IR were shown to induce chromosomal translocations synergistically in LNCaPs by AR - induced chromosomal interactions and DSBs possibly generated by activation - induced cytidine deaminase (AID) and LINE - 1 ORF2 endonuclease (Lin *et al.*, 2009). Knockdown of components of NHEJ-pathway (DNA - PK, Ku70, Ku80, LIG4 and XRCC4) was shown to attenuate, and

removal of ATM or HR proteins (RAD51, XRCC2, RAD52, RAD54 and BRCA1) to enhance the induction of the TMPRSS2 - ERG-fusions, suggesting that NHEJ is the major repair pathway responsible for generating these translocations (Lin *et al.*, 2009). Third, induction of AR target gene expression after androgen stimulation was shown to require TOP2B (Haffner *et al.*, 2010). Moreover, DHT treatment of LNCaP and LAPC4 cells led to the recruitment of AR and TOP2B to regulatory regions of AR target genes and was shown to cause TOP2B-dependent recombinogenic DSBs without extrinsic genotoxic stress (Haffner *et al.*, 2010). Fourth, prolonged androgen stimulation at supra - physiological levels has been confirmed to induce TMPRSS2 – ERG - fusions in immortalized benign prostate epithelial cells, although at a much slower rate (5 months vs. 24 hours) than in malignant cells (Bastus *et al.*, 2010).

1.3. Primary seminal vesicle carcinomas are extremely rare

1.3.1. Seminal vesicle tissue structure and function

Seminal vesicles are a paired, elongated structure located superior to prostate gland (Moore and Dalley, 2006). They secrete alkaline fluid with sucrose and a coagulating agent into the ejaculatory duct. The seminal vesicle arteries and veins are derived form the inferior vesical and middle rectal blood vessels, as are the blood vessels of prostate (Moore and Dalley, 2006). Interestingly, seminal vesicles and the central zone of prostate are embryologically derived from the Wolffian duct and only rarely harbour neoplasms, while the rest of the prostate is derived from the urogenital sinus (McNeal, 1981).

The epithelial structure of seminal vesicles is similar to prostate with a few important exceptions (**Figure 3B**). The basal cell layer in seminal vesicles is discontinuous, the basal cells are more rounded than in prostate and do not express cytokeratin 14 (Laczko *et al.*, 2005). There are no neuroendocrine cells in the seminal vesicles, and secretory cells of seminal vesicles are not positive for PSA or PAP (Laczko *et al.*, 2005). On the other hand, AR is expressed in their secretory and stromal cells and both tissues are androgen dependent (Laczko *et al.*, 2005).

1.3.2. Cancer of the seminal vesicles

True seminal vesicle carcinomas are extremely rare. There were no registered primary seminal vesicle carcinomas (SVCA) in the Finnish Cancer registry during 1953 – 2008 (Risto Sankila, personal communication) and a little more than 50 cases have been described in the medical literature worldwide (Thiel and Effert, 2002). Several diagnostic criteria have been published to distinguish SVCAs from other, more prevalent tumor types

of the anatomical region. First, the tumor should be macro - and microscopically verified carcinoma that is localized exclusively or mainly to seminal vesicles, there must not be other primary carcinomas in the body and the tumor should preferentially be a papillary adenocarcinoma (Dalgaard and Giertzen, 1956). Second, SVCA can be differentiated from prostatic adenocarcinomas, bladder transitional cell carcinomas, rectal and bladder adenocarcinomas and carcinomas of mullerian duct origin by immunohistochemical analysis of five markers: they have been reported to be negative for PSA, PAP and CK 20 and positive for CK7 and CA - 125 (Ormsby *et al.*, 2000). Third, prostate adenocarcinomas have been reported to be negative for mucin 6 that is widely expressed in seminal vesicle epithelium (Leroy *et al.*, 2003).

As they are such an oddity, it is possible that in some rare occasions seminal vesicle tumors are misdiagnosed as one of the secondary tumors originating from prostate, rectum or urinary tract, a much more likely option when tumor growth is detected in seminal vesicles. Nevertheless, there is clearly a significant difference in cancer incidence between prostate and seminal vesicle tissue, which has prompted De Marzo *et al.* (2007) to list this organ selectivity as one of the enigmas that need to be explained in order for us to understand why prostate cancer is so prevalent. Only few reports have attempted to answer this question. Profiling of gene expression from 11 normal prostate and seven seminal vesicle frozen tissue samples indentified 215 candidate cancer genes that were more highly expressed in seminal vesicles, and a subsequent combining of this dataset to another of 1235 genes expressed at lower level in prostate cancer narrowed the number down to a group of 32 genes (Thompson *et al.*, 2008). The identified 32 genes (listed in **Table 1.**) did not affect a single cellular outcome but rather various cellular processes including membrane transport, xenobiotic transport and protease function, and the biologic role of eight genes was unknown (Thompson *et al.*, 2008). Another report recounted that the transgenic adenocarcinoma of mouse prostate (TRAMP) – mice that express SV40 large T antigen under the prostate specific rat probasin promoter, also develop seminal vesicle tumors (Yeh *et al.*, 2009), which indicates that p53 and Rb pathways may have a role in protecting seminal vesicles from tumorigenesis.

Table 1. *List of candidate genes protecting seminal vesicles from carcinogenesis (modified from Thompson et al., 2008). The listed genes were expressed at higher levels in seminal vesicle tissue than in prostate.*

Gene symbol	Chromosome location	Gene name	Biologic process	Additional references
ABCC3	17q22	ATP-binding cassette, sub-family C, member 3	Membrane transport	
ABCG2	4q22	ATP-binding	Membrane	

		cassette, sub-family G, member 2	transport	
C8orf13	8p23.1[a]	Chromosome 8 open reading frame	Unknown, associates with several autoimmune diseases	Hom *et al.*, 2008, Ito *et al.*, 2010a, Ito *et al.*, 2010b, Torres *et al.*, 2010, Nordmark *et al.*, 2011
CAPN6	Xq23	Calpain 6	Protease activity	
CLU	8p21	Clusterin	Possible role in apoptosis	
CRABP2	1q21.3[a]	Cellular retinoic acid binding protein	Retinoic acid signaling	
CXCL6	4q13.3	Chemokine (C-X-C motif) ligand	Chemotaxis	
DNAJC15	13q14.1	DnaJ homolog, subfamily C, member	Unknown, loss may confer drug resistance	Shridhar *et al.*, 2001 Witham *et al.*, 2008
EST	12q12	Expressed sequence tag (AA703625)	Unknown	
EST	4p15.32	Expressed sequence tag (N90470)	Unknown	
FADS2	11q12.2	Fatty acid desaturase 2	Fatty acid metabolism	
FAM46A	6q14	Family with sequence similarity 46, member A	Unknown, may affect 5-FU efficacy in breast cancer	Tsao *et al.*, 2010
FLJ26998	10p13	cDNA FLJ26998	Unknown	
GATA3	10p15	GATA binding protein 3	Lineage-specific transcription	
GLIS3	9p24.2	GLIS family zinc finger 3	Transcriptional regulation	
GOLSYN	8q23.2	Golgi-localized protein	Vesicular transport	
GPRC5B	16p12	G protein-coupled receptor, family C, group 5, member B	Unknown	
GSTM2	1p13.3	Glutathione S-	Xenobiotic	

		transferase M2	metabolism	
GSTP1	11q13	Glutathione S-transferase pi	Xenobiotic metabolism	
KRT7	12q13.13	Keratin 7	Cytoskeletal filament	
LMO3	12p12.3	LIM domain only 3	Unknown, may interact with p53 and reduce its transcriptional activity	Larsen *et al.*, 2010
ME1	6q12	Malic enzyme 1, NADP(?)-dependent, cytosolic	Fatty acid metabolism	
PDK4	7q21.3	Pyruvate dehydrogenase kinase, isozyme 4	Sugar metabolism	
PFKFB3	10p15.1	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	Sugar metabolism	
PTGS2	1q25	Prostaglandin-endoperoxide synthase 2	Prostaglandin synthesis	
PYGL	14q21-q22	Phosphorylase, glycogen	Glycogen metabolism	
SERPINA5	14q32.1	Serpin peptidase inhibitor, clade A, member 5	Coagulation	
SLFN11	17q12	Schlafen family member 11	Unknown	
SLPI	20q12[a]	Secretory leukocyte peptidase inhibitor	Immune response	
SNCG	10q23	Synuclein, gamma	Neurofilament network	
SPINT1	15q15.1	Serine peptidase inhibitor, Kunitz type 1	Protease inhibitor	
TGFBR3	1p33-p32	Transforming growth factor, beta receptor III	TGFbeta signaling	

[a]Prostate cancer susceptibility locus

2. DNA damage response (DDR)

DDR is a common nominator for the activation of multiple pathways after DNA damage. DDR includes detection of the damage and activation of the proper DNA repair pathway, activation of cell cycle checkpoints or decision to enter into apoptosis or senescence. In 2005, two groundbreaking papers described that DDR pathways are activated in premalignant lesions, and suggested that they form a barrier against tumorigenesis (Bartkova *et al.*, 2005; Gorgoulis *et al.*, 2005). They showed that ATM - pathway is constitutively more active in premalignant lesions than in cancer of urinary bladder, breast, colon, lung and skin. The also showed that overexpression of oncogenes that promote replication leads to DDR activation in cancer cell lines. DDR has been confirmed to be active also during prostate tumorigenesis. A study investigating markers of ATM - pathway activation, $ATM^{Ser1981}$, γH2AX and checkpoint kinase 2 (CHK2) Thr68 phosphorylation, of 35 primary prostate cancer and PIN specimens reported that DDR is activated in premalignant PIN lesions more strongly than in cancer (Fan *et al.*, 2006).

The underlying cause of the DDR in premalignant lesions is somewhat debated. It has been proposed that activated oncogenes induce aberrant proliferation and stalling and collapse of replication forks that lead to DSBs especially in common fragile cites (Halazonetis *et al.*, 2008). In premalignant lesions functional DDR activated by DSBs would be able to induce apoptosis or senescence, thus limiting cancer development. In time, constant DNA replication stress could cause genomic instability, inactivate tumor suppressor pathways like ATM and p53 and promote tumorigenesis (Halazonetis *et al.*, 2008).

2.1. ATM – pathway

Ataxia – telangiectasia mutated (ATM) - pathway is essential in orchestrating cellular responses to DSBs. Activation of this pathway has been shown to regulate many stress responses, including DNA damage repair mechanisms, cell cycle checkpoints, cellular proliferation, radiation sensitivity, apoptosis and senescence (Derheimer and Kastan, 2010). An outline of some of the most relevant ATM - mediated responses after DSBs is presented in **Figure 5**.

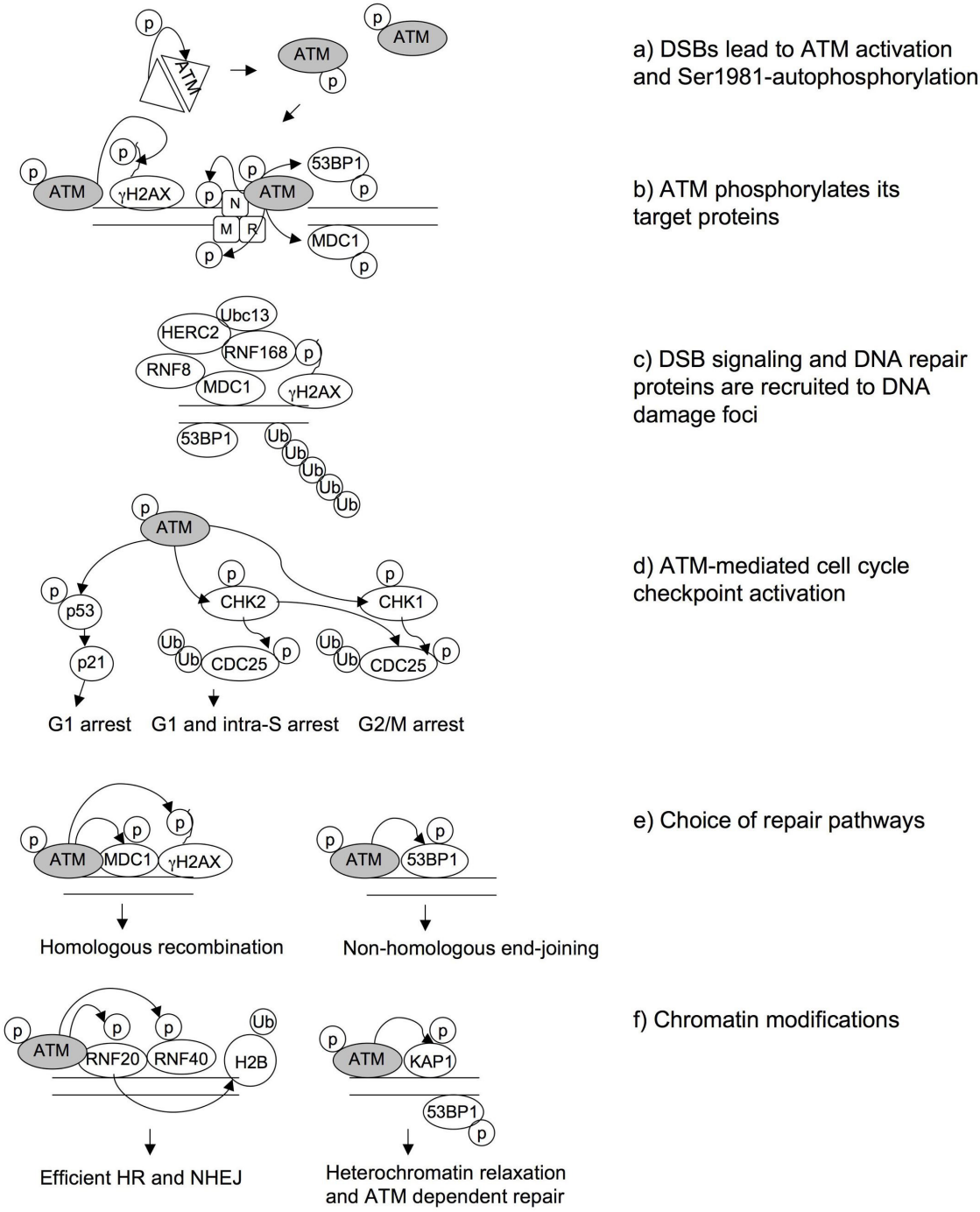


Figure 5. *ATM - pathway activation after DSBs. ATM - kinase activates signaling pathways that regulate DNA damage repair, cell cycle checkpoints and chromatin condensation. See text for details. M,R,N=MRN complex, p=phosphorylation, Ub=ubiquitylation, H2B= histone 2B*

2.1.1. ATM

ATM is one of the key molecules mobilizing cellular response to DSBs. It is a nuclear serine - threonine kinase, which activates other DDR proteins by phosphorylating their serine or threonine residues within SQ or TQ motifs (Derheimer and Kastan, 2010). ATM belongs to the family of phosphatidylinositol - 3 - kinase related protein kinases (PIKKs) together with ATM and Rad3 related protein kinase (ATR) and DNA dependent protein kinase catalytic subunit (DNA - PKcs) mammalian target of rapamycin (mTOR), suppressor of morphogenesis in genitalia (SMG - 1) and transformation / transcription domain - associated protein (TRRAP) (Lempiainen and Halazonetis, 2009). PIKKs are large serine/threonine kinases involved in signaling after cellular stress. DNA - PK is activated by DSBs similarly to ATM, while ATR responds to UV damage, single - stranded DNA and replication stress (Cimprich and Cortez, 2008; Lempiainen and Halazonetis, 2009). ATM is expressed ubiquitously, also in normal prostate tissue and prostate cancer (Angele *et al.*, 2004).

Loss of ATM function causes genomic instability. *Atm* deficient embryonic stem cells are hypersensitive to IR, *Atm* null mouse embryo fibroblasts (MEFs) have been shown to have defective cell cycle checkpoint activation and p53 stabilization after IR and *Atm* null thymocytes are more resistant to IR - induced apoptosis than normal mouse thymocytes (Xu and Baltimore, 1996) (Westphal *et al.*, 1997). Hereditary loss or inactivation of ATM protein function in humans causes a syndrome called A - T. A - T is a recessive autosomal disease that is characterized by a progressive neurological disorder due to cerebellar degeneration (Taylor and Byrd, 2005). The clinical features include ataxia of both upper and lower limbs detectable usually by the age of two, immunodeficiency, growth retardation and telangiectasia (dilated blood vessels that are most noticeable on eyes of the patients). A - T patients are highly sensitive to IR and have a high risk of developing lymphoid tumors with typical chromosomal translocations. Heterozygous patients are usually devoid of these symptoms, but may have slightly increased risk of developing breast cancer (Taylor and Byrd, 2005). *Atm* - deficient mice display similar symptoms as A - T - patients. The *Atm* - / - mice are retarded in growth, have neurological dysfunction, infertility and defects in T - lymphocyte maturation (Barlow *et al.*, 1996; Elson *et al.*, 1996; Xu *et al.*, 1996). They are extremely sensitive to IR and develop thymic lymphomas at an early stage (Barlow *et al.*, 1996; Elson *et al.*, 1996). ATM protein function is not essential for development or viability of these mice (or A - T patients), but the affected tissues indicate that ATM has an important role in maintaining genomic integrity in mitotic, post - mitotic and meiotic cells.

In unstressed cells ATM exists in an inactive, homodimerized state (Bakkenist and Kastan, 2003). After the formation of DSBs, ATM is activated by intermolecular autophosphorylation on serine 1981 and dissociation from the dimers (Bakkenist and Kastan, 2003; Berkovich *et al.*, 2007 and **Figure 5a**). The autophosphorylation of ATM is a

rapid process and has been show to occur even in the absence of DBS by treatments altering chromatin structure, so it is likely that the initial signal activating ATM is a change in the chromatin (Bakkenist and Kastan, 2003). Activated ATM is recruited to the sites of DSBs forming foci that can be visualized by fluorescence microscopy (Andegeko *et al.*, 2001). ATM kinase activity, $ATM^{Ser1981}$ autophosphorylation and functional Nijmegen breakage syndrome protein 1 (Nbs1) protein are required for ATM to accumulate into these DNA damage foci and to initiate DSB repair (Bakkenist and Kastan, 2003; Berkovich *et al.*, 2007; Falck *et al.*, 2005; Wu *et al.*, 2000), although the absolute requirement of serine 1981 phosphorylation has been questioned in mouse models (Pellegrini *et al.*, 2006) and *in vitro* studies (Lee and Paull, 2005). Nbs1 is a part of a complex with meiotic recombination 11 (Mre11) and Rad50 (MRN complex) that accumulates to DSB prior to ATM and is involved in early processing of the break. The presence of MRN complex is required for full ATM kinase activity, retention of ATM at the damaged site and phosphorylation of several downstream targets (Falck *et al.*, 2005; Lee and Paull, 2005; Uziel *et al.*, 2003).

ATM is required for effective DSB repair and it is thought to carry out this function by phosphorylating a vast number of DSB signaling proteins (**Figure 5b**). Some of the ATM target proteins accumulate at the sites of DSBs in a spatially and temporally controlled manner (Bekker - Jensen *et al.*, 2005), while others remain dispersed in the nucleoplasm (Bekker - Jensen *et al.*, 2006). Among the many ATM target proteins that accumulate into sites of DSB and mediate various aspects of DNA repair are Nbs1 (Wu *et al.*, 2000), Mre11, H2AX (Burma *et al.*, 2001; Stiff *et al.*, 2004), mediator of DNA - damage checkpoint 1 (MDC1) (Lou *et al.*, 2006), p53 - binding protein - 1 (53BP1) (Anderson *et al.*, 2001; Rappold *et al.*, 2001; Xia *et al.*, 2001), BRCA1 (Cortez *et al.*, 1999) and KRAB - associated protein (KAP - 1) (Ziv *et al.*, 2006). The role of some of these proteins in DSB signaling will be discussed in more detail in the chapters below. The huge complex of proteins in DNA damage foci is thought to modify chromatin structure and concentrate DNA repair factors into the damaged site so that efficient DSB repair can be accomplished.

Several chromatin modifications have been linked to activation of ATM by DSBs. One of the most well - known, phosphorylation of histone variant H2AX on serine 139 (γH2AX), will be discussed in more detail below. Activation of the ATM - pathway has been shown to lead to the removal of histone 2B (H2B) and accumulation of XRCC4 in cells synchronized into G1 (Berkovich *et al.*, 2007). Another target of ATM, nuclear phosphoprotein KAP - 1 is known as a repressor of transcription, that is associated with heterochromatin formation. Around 10 – 25 % of DSBs in nondividing cells are defectively repaired if ATM signaling is lost, and this occurs specifically in the regions of heterochromatin (Goodarzi *et al.*, 2008; Riballo *et al.*, 2004). It has been shown that ATM - dependent KAP - 1 phosphorylation on serine 824 mediates heterochromatin relaxation, increases cell survival after DSBs and is thought to make damaged DNA more accessible for repair factors (Goodarzi *et al.*, 2008; Ziv *et al.*, 2006). Recently, ATM was shown to phosphorylate heterodimeric E3 ubiquitin ligase complex formed by RNF20 and RNF40,

which induced monoubiquitylation of histone H2B in an ATM - dependent manner (Moyal *et al.*, 2011). This modification has been associated with transcription elongation in unstressed cells, but after DSBs it was required for optimal repair of DSBs by NHEJ and HR (Moyal *et al.*, 2011).

Presence of DSBs needs to be signalled to the cell cycle checkpoint machinery in order for cells to avoid DNA replication and mitosis with damaged chromosomes. ATM signaling has been shown to affect all the cell cycle checkpoints, which will be discussed in more detail in the chapter on cell cycle checkpoints. Further roles of ATM include telomere maintenance (Celli and de Lange, 2005; Karlseder *et al.*, 2004) and insulin signaling (Schneider *et al.*, 2006; Yang and Kastan, 2000).

2.1.2. H2AX

DNA in cell nucleus is packed around histone proteins to form the organized structure of chromatin. The core unit of chromatin, the nucleosome, consists of four types of core histone proteins, H2A, H2B, H3 and H4 and a linker histone H1 acting as a bridge between the nucleosomes. All the core histones are covalently modified by a wide variety of enzymes to regulate processes like transcription, DNA repair or apoptosis (Jenuwein and Allis, 2001). These covalent modifications can either alter the state of chromatin compaction or binding of other proteins to DNA. All the core histones are covalently modified in response to DSBs and these modifications have an integral role in DDR and DSB repair (Downs *et al.*, 2007).

Histone H2AX is a variant form of the core histone H2A. It has been estimated that H2AX constitutes 2 – 20 % of the total H2A pool and is found on average in every fifth nucleosome (Bonner *et al.*, 2008). Most core histones are synthesized during S - phase of the cell cycle, but H2AX can also be transcribed in a replication - independent manner (Mannironi *et al.*, 1989; Wu *et al.*, 1982). It has an evolutionary conserved C - terminal tail that protrudes out of the globular structure of histones and thus is accessible to modifying proteins (Dickey *et al.*, 2009). Since 1998, when DSBs were reported to induce H2AX phosphorylation on serine 139 (Rogakou *et al.*, 1998) and specific antibodies could be used to detect this γH2AX in DNA damage foci (Rogakou *et al.*, 1999), γH2AX has been widely used as a sensitive marker for DSBs (Bonner *et al.*, 2008; Dickey *et al.*, 2009 and **Figure 6.**). Although the bulk of attention has been focused on γH2AX, there are at least two other phosphorylation sites on H2AX (Cook *et al.*, 2009; Pantazis and Bonner, 1981; Xiao *et al.*, 2009).

Hereditary syndromes with genomic instability involving mutations of H2AX gene have not been described to date, but the physiological consequences of H2AX loss have been investigated in H2AX deficient mice (Celeste *et al.*, 2002). H2AX null mice share many but not all features of *Atm* - / - mice. They are viable, growth retarded, immune deficient,

sensitive to irradiation, and the males are infertile (Celeste *et al.*, 2002). H2AX -/- MEFs grow poorly in culture and embryonic stem cells (ES), MEFs and T - cells exhibit a high number of chromosomal aberrations, although contrary to *Atm* -/- cells, they do not have explicit defects in cell cycle checkpoints (Bassing *et al.*, 2002; Celeste *et al.*, 2002). The recruitment of Nbs1, 53BP1 and Brca1 to IR induced DNA damage foci is impaired in H2AX -/- ES cells, B - cells and fibroblasts (Bassing *et al.*, 2002; Celeste *et al.*, 2002). Moreover, deletion of a single H2AX allele has been show to increase the number of chromosomal translocations and to enhance tumorigenesis in a p53 null background in mice, indicating that maintenance of sufficient levels of H2AX are required for achieving optimal DSB repair (Bassing *et al.*, 2003; Celeste *et al.*, 2003a). ATM and H2AX have significantly overlapping functions in DSB repair, especially in G1 phase of the cell cycle, as combined *Atm* -/- *H2AX* -/- deficiency leads to embryonic lethality and severe genomic instability in ES cells, MEFs and mature B and T cells (Zha *et al.*, 2008). The ATM independent functions of H2AX may include repair of postreplication DNA damage (Zha *et al.*, 2008).

H2AX is phosphorylated in response to many types of DNA damaging agents (Takahashi and Ohnishi, 2005), and the primary kinases carrying out the γ - phosphorylation are ATM, ATR and DNA–PK. Distinct types of DNA lesions activate the three PIKKs (Burma *et al.*, 2001; Park *et al.*, 2003; Stiff *et al.*, 2004; Ward and Chen, 2001; Ward *et al.*, 2004). ATR is sensitive to UV lesions and replication stress and induces a more diffuse pan - nuclear H2AX phosphorylation, while AMT and DNA - PK detect DSBs and result in a focal staining pattern of γH2AX (Marti *et al.*, 2006). Although both ATM and DNA - PK are able to phosphorylate H2AX redundantly in response to DSBs (Stiff *et al.*, 2004), it seems that ATM is the major kinase phosphorylating γH2AX after IR (Burma *et al.*, 2001) and DNA - PK, as a part of the NHEJ - pathway, is more involved in the repair of the lesions (Koike *et al.*, 2008; Mahaney *et al.*, 2009). There also may be some tissue specificity between ATM and DNA - PK in respect of γH2AX (Koike *et al.*, 2008).

The role of H2AX in DSB repair is mainly mediated by its γ - phosphorylation. γH2AX foci serve as platforms for the recruitment of several DNA repair and chromatin remodelling factors (e.g. Nbs1, Rad50, 53BP1 and Brca1) needed for the DSB signaling (Celeste *et al.*, 2003b; Fernandez - Capetillo *et al.*, 2003; Paull *et al.*, 2000). Also, γH2AX is reported to be involved in the G2/M cell cycle checkpoint activation after low amounts of DNA damage (Fernandez - Capetillo *et al.*, 2002), in recruitment of cohesins to promote sister chromatid - dependent recombinatiorial repair (Xie *et al.*, 2004), and in prevention of dissociated of broken DNA ends (Bassing and Alt, 2004). Besides the rapid γH2AX induction, successful DSB repair seems also to require timely removal of the γH2AX signal. Several phosphatases, including PP2A and PP4 have been implicated in the dephosphorylation of γH2AX (Chowdhury *et al.*, 2005; Chowdhury *et al.*, 2008).

There are two phosphorylation events of H2AX that have a role in apoptosis. First, γH2AX has long been known to be induced by various treatments causing apoptosis and to

coincide with the initiation of DNA fragmentation, an ultimate form of DSBs (Rogakou *et al.*, 2000). The apoptotic γH2AX signal is pan - nuclear and intensive, and may be mediated by other kinases than DNA - PK or ATM (Mukherjee *et al.*, 2006). One of the kinases phosphorylating γH2AX after apoptotic dose of UV damage has been reported to be c - Jun N - terminal kinase (JNK) (de Feraudy *et al.*, 2010; Lu *et al.*, 2006), and γH2AX under these circumstances was required for efficient DNA ladder formation (Lu *et al.*, 2006). Second, H2AX phosphorylation on another residue, tyrosine 142 has been shown to regulate DNA repair, apoptosis and survival decisions (Lukas and Bartek, 2009). Tyrosine142 phosphorylation levels are maintained constant in unstressed cells by tyrosine kinase WSTF, which has previously been implicated in nucleosome mobilization and DNA replication (Xiao *et al.*, 2009). After DNA damage, the levels of tyrosine 142 decrease concomitantly with increased γH2AX, but without prior WSTF activity and tyrosine 142 phosphorylation, γH2AX cannot be maintained and ATM and MDC1 recruitment to DSBs are compromised (Xiao *et al.*, 2009). The phosphatases that remove tyrosine 142 phosphorylation from H2AX after DNA damage have been identified as EYA1 and EYA3 that are known to regulate organ development. If tyrosine 142 is removed by EYA, γH2AX is able to recruit MDC1 and promote DNA damage repair. If both phosphorylations are present, MDC1 accumulation to DSBs is inhibited and JNK1 is able to bind to tyrosine 142 and drive apoptosis (Cook *et al.*, 2009). How this apoptotic switch is orchestrated in human cells still needs more investigation, as we currently do not know what determines the balance between the serine 139 and tyrosine 142 phosphorylations after DSBs.

H2AX deficient mice have indicated that H2AX is important in tumor suppression and maintenance of genomic integrity (Bassing *et al.*, 2002; Bassing *et al.*, 2003; Celeste *et al.*, 2002; Celeste *et al.*, 2003a). Clearly H2AX has a role in mediating recruitment of DSB signaling and repair proteins onto damaged chromatin, and it may be involved in deciding between DNA damage repair and apoptosis. How important these H2AX functions are for the tumor suppressor function of H2AX has not been fully addressed, and it remains to be determined in which cell types, phases of cell cycle and under what circumstances H2AX function cannot be overlooked.

2.1.3. 53BP1

53BP1 was first described through its ability to bind to tumor suppressor p53, and it was suggested to enhance p53 - mediated transcription (Iwabuchi *et al.*, 1994; Iwabuchi *et al.*, 1998). Then it was recognized as a part of DSB signaling complex that is phosphorylated by ATM and accumulates onto chromatin at sites of DSBs (Anderson *et al.*, 2001; Rappold *et al.*, 2001; Schultz *et al.*, 2000; Xia *et al.*, 2001), and started to attract more attention.

Generation of 53BP1 – deficient mice indicated that it is required for genomic integrity and has a role in tumor suppression (Morales *et al.*, 2003; Ward *et al.*, 2003). Similarly to

Atm and *H2AX* deficient mice, 53BP1 null mice were born at expected rates, were growth retarded, had immunodeficiencies and were sensitive to irradiation, chromosomal abnormalities and thymic lymphomas. However, contrary to *Atm* -/- mice, they had only very mild cell cycle checkpoint defects and were fertile, which indicates that 53BP1 is not essential in meiosis (Morales *et al.*, 2003; Ward *et al.*, 2003). Further, the number of spontaneous chromosomal aberrations is lower in 53BP1 -/- cells and the increase in tumor incidence only mild, which findings are compatible with the role of 53BP1 downstream of ATM. Loss of p53 combined with 53BP1 deficiency greatly increases lymphomagenesis in mice and the number of chromosomal abnormalities, but conflicting reports have been published on whether there is dosage - dependency (haploinsufficiency) in 53BP1 - mediated tumor suppression (Morales *et al.*, 2006; Ward *et al.*, 2005).

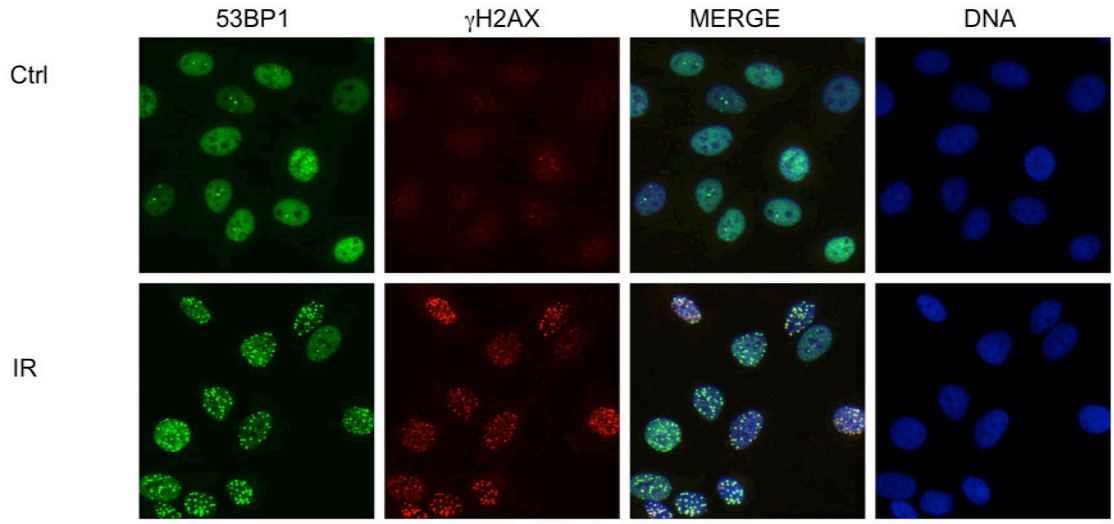


Figure 6. *γH2AX induction and 53BP1 relocalization from nucleoplasm into DNA damage foci after IR in U2OS osteosarcoma cell line.*

In unstressed cells, 53BP1 is nucleoplasmic in G1, accumulates into foci in S, and is expressed at very low levels in G2/M phase (Morales *et al.*, 2003) and **Figure 6**. As it was known to interact with a well - known cell cycle regulator, p53, 53BP1 was also suspected to play a role in checkpoint activation after DNA damage. Depletion of 53BP1 from human cancer cell lines or mouse B - cells and MEFs was shown to result in defective intra - S - phase and G2/M checkpoints with low (2 - 3 Gy) doses of IR (Fernandez - Capetillo *et al.*, 2002; Wang *et al.*, 2002) and to affect p53 stability and Chk2 phosphorylation (Wang *et al.*, 2002). On the other hand, others have reported that the S - and G/M checkpoint defects in mouse embryonic cells are slight and could rather be caused by impaired DSB repair than a bona fide cell cycle checkpoint defect (Ward *et al.*, 2003).

53BP1 protein has no characterized enzymatic activity that could explain its role in DSB repair. Instead, it is considered to act as a recruitment platform for other DDR proteins (FitzGerald *et al.,* 2009). Accumulation and retention of 53PB1 in DSB foci is complex and requires the activity of several ubiquitylating enzymes and methylated histones. First, γH2AX has been shown to be dispensable for the initial recruitment of 53BP1 to the DSB, but required for its sustained retention at the sites (Celeste *et al.*, 2003b). Second, MDC1 seems to be required either directly (Eliezer *et al.,* 2009) or indirectly via other post - translational modifications. Phosphorylated MDC1 interacts with γH2AX and a E3 ubiquitin ligase RNF8 (Mailand *et al.*, 2007), which forms a complex with E2 conjugating enzyme UBC13 and a candidate assembly factor HERC2 (Bekker - Jensen *et al.*, 2010) and ubiquitylates H2A. This ubiquitinylated H2A then interacts with another ubiquitin ligase RNF168 that together with UBC13 amplifies the lysine - 63 ubiquitin conjugation at DSBs (Doil *et al.,* 2009). The MDC1 - RNF8 - RNF168 - HERC2 –mediated polyubiquitylation seems to be essential for successful retention of 53BP1 at DSBs. Third, 53BP1 has also been reported to interact with methylated histone residues. Structural analyses on the tandem tudor domain of 53BP1 indicated that histone H3 methylated on lysine 79 (H3 - K79me) is able to bind to 53BP1 (Huyen *et al.*, 2004). H3 - K79me is mediated by a methyltransferase DOT1L, and downregulation of DOT1L decreases the number of 53BP1 foci after IR. As H3 - K79 methylation levels do not change after IR, it has been suggested that DSBs alter nucleosome stacking so that this histone mark is exposed supporting 53BP1 binding (Huyen *et al.*, 2004). On the other hand, another report rejected that H3 - K79me would have a significant biological role in recruitment of 53BP1 and instead presented that direct interaction between 53BP1 and histone H4 lysine 20 dimethylation (H4 - K20me2) is required for 53BP1 retention at DSBs (Botuyan *et al.*, 2006). Regulation of H4 - K20me2 has recently been connected to ATM activity, as H4 - K20me2 levels were reportedly locally induced by a new histone methyltransferase MMSET, another ATM - target that is recruited to DSBs in an γH2AX - MDC1 – dependent manner (Pei *et al.*, 2011).

However 53BP1 may be recruited to DSBs, it has a role in protecting chromosomal integrity in response to IR. Recent reports indicate that 53BP1 function seems to be required especially in NHEJ and repair of heterochromatin DSBs. As NHEJ and HR both repair DSB, inhibition of one leads to increased need of the other one. Depletion of 53BP1 or preventing its interaction with H4 - K20me2 was shown to increase HR levels in an XRCC4 dependent manner (Xie *et al.*, 2007). Interestingly, this 53BP1 - mediated NHEJ was reported to be independent of H2AX (Xie *et al.*, 2007). Another study indicated that 53BP1 and its interaction with H4 - K20me2 are required for optimal NHEJ of unprotected telomeres (Dimitrova *et al.*, 2008). They suggested that 53BP1 increases chromatin mobility that is needed to bring the damaged DNA ends into close proximity (Dimitrova *et al.*, 2008). 53BP1 has also been linked to the ATM - dependent KAP - 1 phosphorylation that is essential for repair of DSBs in heterochromatin regions (Noon *et al.*, 2010).

2.1.4. DSB signaling network involves a complex array of post - translational modifications

The cellular signaling occurring after DSBs is complex and involves many other post - translational modifications and proteins than discussed above. Merely the number of phosphorylation events induced by DSBs is astounding. Extensive characterization of IR - induced phosphorylations on SQ - TQ –motifs, which are common in substrates for ATM (Kastan and Lim, 2000) and ATR, identified around 900 phosphorylation events in over 700 proteins, most of which had not been previously described (Matsuoka *et al.*, 2007). Based on their biological function, 420 proteins out of the 700 could be divided into various categories, including highly relevant groups of DNA replication, recombination and repair (46 genes), cell cycle (72 genes), mRNA transcription (101 genes) and chromatin remodelling (22 genes). ATM was estimated to regulate 70 % of the substrates. Another group investigating both ATM - dependent and - independent phosphorylation events occurring after DSBs could identify 411 damage - induced phosphorylations and 342 dephosphorylations on 394 proteins (Bensimon *et al.*, 2010). Approximately 60 % of these were ATM - dependent, supporting the role of ATM as the main transducer of DSB response. Among the other kinases involved were ATR, DNA - PK, cyclin - dependent kinase 1 (CDK1), cyclin - dependent kinase 2 (CDK2) and casein kinase 2 (CK2).

CDK1 and CDK2 have an important role in cell cycle checkpoints, and they will be discussed below. The role of CK2 in DSB repair is not characterized in detail, but it has been connected to the ever - growing group of DSB – induced chromatin modifiers (Lukas and Bartek, 2008). CK2 has been reported to be required for phosphorylation of heterochromatin protein 1 - β (HP1 - β) after DSBs (Ayoub *et al.*, 2008). In unstressed cells HP1 - β is bound to histone H3 methylated on lysine 9 (H3K9me). Phosphorylation of HP1 - β after DNA damage releases it from chromatin, and this mobilization of HP1 - β enables efficient phosphorylation of γH2AX (Ayoub *et al.*, 2008). In addition, CK2 has been shown to phosphorylate H4 serine 1 after DNA damage (Cheung *et al.*, 2005) and facilitate repair of single strand breaks (Loizou *et al.*, 2004).

Phosphorylation is only one of the many possible post - translational modifications occurring after DNA damage. As discussed above, ubiquitylation events may be equally important. Ubiquitylation requires activity of three different types of enzymes: E1 ubiquitin - activating enzyme activates ubiquitin so that it can be transferred to its target proteins by E2 ubiquitin - conjugating enzymes and E3 ubiquitin - ligases (Chen and Sun, 2009). Several DSB signaling proteins, including RNF8, RNF168, Ubc13 and BRCA1, are E2 and E3 enzymes (Messick and Greenberg, 2009; Panier and Durocher, 2009). Respectively, their less studied counteracting enzymes, deubiquitinating enzymes (DUBs) are able to modify DSB signaling (Nakada *et al.*, 2010). There are also reports indicating that acetylation (Fischle, 2009), methylation and sumoylation events (Bartek and Hodny, 2010; Galanty *et al.*, 2009; Morris *et al.*, 2009) are closely involved in DSB repair. It still remains

unresolved how all these signaling routes are coordinated (Ciccia and Elledge, 2010), but chromatin structure and the organization of the genome have an effect (Misteli and Soutoglou, 2009). We are still far from understanding the complete picture of DDR.

2.2. p53, the guardian of the genome

p53 tumor suppressor is essential for maintenance of genomic integrity. It has been under rigorous investigation for more than 30 years, and its role in tumor suppression has proven to be rather complex (Vousden and Prives, 2009; Zilfou and Lowe, 2009). It is generally accepted that p53 is a transcription factor and regulates multiple processes implicated in cancer, including cell cycle checkpoints, cellular senescence, autophagy and apoptosis (Zilfou and Lowe, 2009). It is activated by various stress signals, including DNA damage, oncogene activation, ribosomal stress, loss of cell - cell contacts and hypoxia (Horn and Vousden, 2007). Considering that p53 is mutated in approximately 50 % of human tumors and in a large proportion of the rest p53 pathway is inactivated by another means, p53 has been an attractive target for new cancer drug development (Brown *et al.*, 2009).

Germ - line p53 mutations result in a familial cancer syndrome called Li - Fraumeni that is characterized with early - onset tumors (Malkin *et al.*, 1990). The classical Li - Fraumeni component tumors are breast carcinomas, bone and soft tissue sarcomas, brain tumors, adrenocortical carcinomas and leukemias that account for approximately 75 % of all the tumors detected in Li - Fraumeni (Nichols *et al.*, 2001). The remaining 25 % originate from diverse sites, but tumors of lung, stomach, ovaries, colon and rectum have been reported on multiple occasions. In a study listing 738 tumors of 185 Li - Fraumeni - patients and their first - degree relatives, prostate cancer was detected in 7 cases corresponding to 0,1 % of all tumors (Nichols *et al.*, 2001). This clearly indicates that in prostate tumorigenesis p53 has a limited role. Similar results have been obtained from p53 null mice. They are prone to a wide spectrum early - onset tumors, especially to lymphomas and sarcomas (Donehower *et al.*, 1992; Harvey *et al.*, 1993). IR is able to decrease tumor latency of heterozygous but not p53 null mice, where spontaneous tumor rate is already high (Kemp *et al.*, 1994).

Due to its potent growth inhibitory effects, p53 levels in unstressed cells are kept low by proteosomal degradation. Under normal circumstances, two structurally related proteins murine double minute - 2 (Mdm2) and Mdm2 related protein (MDM4, also known as MdmX) are essential for suppression of p53 activity (Marine *et al.*, 2006). Mdm2 is the major E3 ubiquitin ligase that ubiquitinates p53 and directs it into degradation (Haupt *et al.*, 1997; Honda *et al.*, 1997; Kubbutat *et al.*, 1997). Transcription of Mdm2 is directly activated by p53, and this forms a negative feedback loop that is essential for keeping p53 activity under control. MDM4 is responsible for transcriptional repression of p53 (Marine and Jochemsen, 2004). A long list of other E3 ligases, including Cop1 (Dornan *et al.*, 2004)

and Pirh2 (Leng *et al.*, 2003) have been described for p53, but they are probably not as essential as Mdm2.

p53 is stabilized and activated rapidly after DNA damage through a series of post - translational modifications that release it from its negative regulators (Lavin and Gueven, 2006). There are more than 36 sites of p53 that can be phosphorylated, acetylated, methylated, ubiquitinated or sumoylated by a variety of different proteins (Kruse and Gu, 2009). p53 is phosphorylated at least by ATM (Canman *et al.*, 1998; Saito *et al.*, 2002), ATR (Tibbetts *et al.*, 1999), DNA - PK, JNK, CK2, checkpoint kinase 1 (CHK1) and CHK2 (Chehab *et al.*, 2000), all of which are implicated in DDR. IR induces ATM mediated phosphorylations either directly or via CHK2 (Chehab *et al.*, 2000) on multiple sites of p53 and Mdm2 (Maya *et al.*, 2001) and destabilizes their interaction. Also MDM4 is phosphorylated in the process.

p53 stability and activity are further modulated by two DUBs called herpesvirus - associated ubiquitin - specific protease (HAUSP) and USP10. HAUSP is able to deubiquitinate and stabilize p53 (Li *et al.*, 2002), but in unstressed cells it also stabilizes Mdm2 and Mdm4 favouring p53 degradation. ATM - mediated phosphorylation of Mdm2 and Mdm4 lowers their affinity towards HAUSP and shifts the balance towards p53 stabilization after IR (Meulmeester *et al.*, 2005). USP10 is a cytoplasmic ubiquitin specific protease that stabilizes p53 and counteracts Mdm2 - mediated degradation (Yuan *et al.*, 2010). After IR, USP10 is phosphorylated by ATM, is stabilized and translocates into the nucleus, where it further increases p53 activity (Yuan *et al.*, 2010). How p53 is stabilized *in vivo* in different tissues and by various forms of stress has still not been completely resolved (Kruse and Gu, 2009).

p53 regulates many of its biological functions by inducing transcription of a suitable set of genes (Espinosa, 2008). There are at least 129 potential p53 responsive genes in the human genome (Riley *et al.*, 2008) and considerable tissue and cell specificity exists in expression of p53 target genes both basally and after genotoxic treatments (Bouvard *et al.*, 2000; Butz *et al.*, 1998; Fei *et al.*, 2002; Komarova *et al.*, 1997; Komarova *et al.*, 2000). For example, p21 has been shown to be expressed basally in mouse small intestine, lung and heart, while proapoptotic BAX was expressed also in spleen, thymus and brain tissue (Bouvard *et al.*, 2000). Mouse spleen and thymus showed a high induction of p21 after IR as did heart, lung, liver and brain. BAX was mostly activated in thymus and spleen that are known to be radiosensitive organs (Bouvard *et al.*, 2000).

There are two ways for p53 to induce apoptosis: either directly or by inducing transcription of proapoptotic genes. p53 can translocate into mitochondria, induce mitochondrial outer membrane permeabilization and release proapoptotic factors. The proapoptotic genes upregulated by p53 include BAX, PUMA, NOXA, p53AIP and PIG3 (Vousden and Lu, 2002). p53 and ATM have been show to cooperate in induction of apoptosis in mouse thymocytes (Westphal *et al.*, 1997). If both *TP53* and *Atm* are deleted,

tumorigenesis is accelerated and the tumor spectrum is broader than with a loss of a single gene (Westphal *et al.*, 1997).

It is difficult to pinpoint the most relevant tumor suppressive function of p53, as the effects of p53 induction are dependent on many factors including cell type and genetic background (Zilfou and Lowe, 2009). Nevertheless, several mouse models have been used to investigate this question in a particular setting (Lozano, 2010). There are some indications that ATM mediated p53 activation is required (Meek, 2009). At first it was reported that the lack of serine 18 phosphorylation (mouse equivalent for serine 15) would not render mice susceptible to tumors (Chao *et al.*, 2003), but later analysis on the mice indicated that they are prone to several late - onset malignancies, including lymphomas and sarcomas (Armata *et al.*, 2007). Mice where both p53 serine 18 and 23 (corresponding to human serine 15 and 20) are changed into alanines and cannot be phosphoyrlated, develop a spectrum of tumors that differs from p53 -/- mice (Chao *et al.*, 2006). A recent DNA sequencing study on 188 human lung adenocarcinomas revealed that ATM and p53 mutations were mutually exclusive, suggesting that the loss of either one is sufficient to silence the DDR pathway (Ding *et al.*, 2008).

On the other hand, there are reports that indicate the opposite. It has been postulated that oncogenic stress stabilizes Arf by preventing its degradation and that this activates p53 - mediated growth arrest and mediates most of p53 tumor suppression (Chen *et al.*, 2010; Martins *et al.*, 2006), while p53 response induced by DNA damage may be of less importance. One study used a conditional mouse model where p53 activity can be switched on and off to study the role of acute DDR in suppression of IR - induced lymphomas (Christophorou *et al.*, 2006). They concluded that if p53 was allowed to function immediately after IR, it caused widespread apoptosis of lymphoid tissues and intestinal epithelium, but did not succeed in reducing lymphoma incidence from p53 null mice. If p53 activity was restored eight days after IR, the acute toxic effects were abrogated and IR - induced lymphomas were suppressed in an Arf - dependent manner (Christophorou *et al.*, 2006). p53 mediated cellular senescence has been implicated in mouse prostate tumorigenesis. Conditional inactivation of p53 alone did not result in prostate tumors, but combined loss of PTEN and p53 led to rapid carcinogenesis (Chen *et al.*, 2005). This was explained by the fact that PTEN loss induced high levels of Arf, p53 and p21, and abrogation of p53 abolished this senescence response and allowed rapid progression of tumors (Chen *et al.*, 2005).

2.3. Cell cycle checkpoints

DNA damage can transiently delay cell cycle progression in G1, S and G2/M phases through inhibition of CDK activity. The main cyclin - CDK - pairs governing the checkpoints are CDK2 and cyclin E for G1, CDK2 and cyclin E/A for S - phase

(Malumbres and Barbacid, 2009), and CDK1 and cyclin B for G2/M (Lindqvist *et al.*, 2009). ATM and ATR have a paramount role in launching a cascade of rapid phosphorylation events that leads to the inhibition of CDK/cyclin complexes and subsequent checkpoint activation after DNA damage (Iliakis *et al.*, 2003). The phosphorylation cascade involves early DNA damage sensors (e.g. MRN complex, ATM, ATR), mediators (e.g. MDC1, 53BP1) and transducers (e.g. checkpoint kinase 1 (CHK1) and CHK2) of the signal, and effector molecules (e.g. p53, cell division cycle 25 phosphatase (CDC25)) that modulate cellular function (Kastan and Bartek, 2004; Lobrich and Jeggo, 2007). ATM and CHK2 are expressed throughout the cell cycle and also in quiescenct or differentiated cells, while ATR and CHK1 are restricted to S - G2 - phases (Jones *et al.*, 2004; Kaneko *et al.*, 1999; Lukas *et al.*, 2001; Matsuoka *et al.*, 1998). In the following chapters the main focus is on how IR and DSB activate the G1, intra - S and G2/M checkpoints.

2.3.1. G1 checkpoint

G1 - checkpoint prevents cells with damaged DNA from progressing from G1 into S - phase. It is thought to be more sensitive than the G2/M checkpoint, and has been proposed to be the master regulator of genomic stability (Lobrich and Jeggo, 2007). There are two partially overlapping mechanisms that activate G1 arrest: a rapid, transient and p53 - independent pathway and a slower, more permanent p53 - mediated arrest that requires transcription of new proteins (Bartek and Lukas, 2001; Kuerbitz *et al.*, 1992). Both are ATM - dependent. In the first pathway, activated ATM phosphorylates CHK2 that in turn phosphorylates phosphatase CDC25A. This increases the proteolytic degradation of CDC25A (Mailand *et al.*, 2000) and thus prevents the removal of inhibitory threonine 14 and tyrosine 15 phosphorylations from CDK2. The ATM - CHK2 - CDC25A - CDK2 pathway is activated within 20 - 30 minutes after IR, and during the first hours is able to reduce the number of cells entering into S - phase (Deckbar *et al.*, 2010). The second, more permanent G1 arrest –pathway is also initiated by ATM, which phosphorylates p53 on its amino terminus serine 15 (Banin *et al.*, 1998; Canman *et al.*, 1998). As discussed above, this and further modifications of negative regulators of p53, such as Mdm2, MDM4 and USP10, release p53 from degradation and increase its ability to transactivate gene expression. The key transcriptional target of p53 in G1 arrest is the CDK inhibitor p21 (Abbas and Dutta, 2009). In some cell types, e.g. in normal human fibroblasts, IR - triggered long - term induction of p21 and a prolonged G1 growth arrest may be reminiscent of cellular senescence (Di Leonardo *et al.*, 1994). In others, G1 arrest is not permanent.

G1 checkpoint is sensitive and has been estimated to arrest cell cycle progression even after a single DSB (Huang *et al.*, 1996), but it is by no means flawless. In fact, there are two

significant limitations to the G1 checkpoint (Deckbar *et al.*, 2010). First, the initial CHK2 - dependent transient arrest does not fully abolish S - phase entry, and full G1 arrest is only observed several (>4) hours after IR. Second, the G1 - checkpoint is maintained inefficiently, as a small fraction of cells (<5 %) is able to progress into G2 with markers of DNA damage. Both limitations can lead to appearance of chromosomal breaks and have the potential to cause genomic instability.

G1 to S - phase progression is altered in a significant number of prostate cancer cases. Loss of chromosome 13q and RB and CDK4 inhibitor p27 expression are both prevalent in prostate tumors (De Marzo *et al.*, 1998; Abate - Shen and Shen, 2000). More recently, integrated oncogenomic profiling showed that the regulation of RB signaling is affected by mutations, copy - number alterations and expression changes of p27, cyclin E1, CDK2 and RB in 34 % of primary prostate tumors (Taylor *et al.*, 2010). In addition, the DNA damage activated G1 checkpoint may be deregulated at least in a subset of prostate tumors, as germline mutations in the gene encoding CHK2 have been reported in sporadic (in 4,8 % of cases) and familial (3,3 – 10,8 %) prostate cancer cases (Dong *et al.*, 2003; Seppälä *et al.*, 2003). At least two of these mutations, 1100delC and IVS2+1G→A reduce the levels of CHK2 expression (Dong *et al.*, 2003). The role of p53 in the maintenance of the G1 checkpoint in prostate cancer is more ambiguous. Missense mutations of *TP53* are rare in early stages of prostate cancer, but copy - number loss is much more prevalent, detected in ca. 24 % of cases (Taylor *et al.*, 2010).

2.3.2. Intra - S - checkpoint

Lack of intra - S - phase checkpoint activation results in radioresistant DNA synthesis (RDS), a feature long connected to cells derived from AT - patients (Painter, 1981). Intra - S - phase checkpoint is governed by ATM and ATR and shares many features with the G1 arrest (Lukas *et al.*, 2004). Activation of the checkpoint results in an arrest that lasts only for a few hours. It consists of the ATM - CHK2/ATR - CHK1 - CDC25A phosphorylation cascade. Phosphorylated CHK1 or CHK2 phosphorylate CDC25A on its serine 123 and direct it to degradation, which allows the retention of the inhibitory threonine 14 and tyrosine 15 phosphorylations on CDK2 (Falck *et al.*, 2001). Inhibition of CDK2 blocks the loading of CDC45 onto chromatin and prevents recruitment of DNA polymerase. The degradation of CDC25A is already maximal 30 minutes after IR, and gradually recovers to basal levels in four - eight hours. Normally, DNA - synthesis is reduced by 50 % few hours after a dose of 10 Gy of IR (Falck *et al.*, 2001).

Many other proteins have been implicated in the maintenance of the intra - S - checkpoint but their function is not entirely clear. It is possible that the mediators of the DSB signaling implicated in cell cycle checkpoints including MDC1 (Goldberg *et al.*,

2003), BRCA1 and 53BP1 (Wang *et al.*, 2002), are required to amplify the signaling cascade to the level that is sufficient in launching the arrest.

2.3.3. G2/M checkpoint

G2/M checkpoint prevents mitotic entry with damaged DNA. It has been suggested to be less sensitive than the G1 checkpoint and to tolerate 10 or even 20 DSBs before activation (Deckbar *et al.*, 2007; Lobrich and Jeggo, 2007). It is mostly p53 independent, as many tumor cell lines with inactivated p53 still maintain functional G2/M checkpoint. Similarly to the rapid activation of G1 and intra - S - arrests, G2/M checkpoint is mediated by CHK1 and CHK2, but the pathway is somewhat more complex. In G2, both ATM - CHK2 and ATR - CHK1 activate the degradation of CDC25A and cytoplasmic sequestration of its family members CDC25B and CDC25C. This leads to inhibition of CDK1/cyclinB activity.

2.3.4. Role of Wee1A in cell cycle checkpoints

Wee1A tyrosine kinase phosphorylates CDK1 and CDK2 on tyrosine 15 and inhibits their kinase activity (Parker and Piwnica - Worms, 1992; Watanabe *et al.*, 1995). Another, related kinase called Myt1 preferentially phosphorylates CDK threonine 14 residues (Liu *et al.*, 1997; Mueller *et al.*, 1995). Wee1 and Myt1 counteract the effects of CDC25 phoshatase family members that remove these inhibitory phosphorylations (Parker and Piwnica - Worms, 1992). Together they all form the inner feedback loop regulating cyclin B – CDK1 complex activity that is important for normal cell cycle coordination as well as G2/M DNA damage checkpoint (Lindqvist *et al.*, 2009).

Wee1 is located predominantly in the nucleus (Baldin and Ducommun, 1995; McGowan and Russell, 1995), while CDK1 - cyclin B complex and CDC25 phosphatase family members can shuttle back and forth between nucleus and cytoplasm. Myt1 localizes to the endoplasmic reticulum and Golgi (Liu *et al.*, 1997). Wee1 kinase activity and cellular levels are low in G0/G1, start to increase in S - phase and remain high until the onset of mitosis (Baldin and Ducommun, 1995; McGowan and Russell, 1995; Watanabe *et al.*, 1995). Multiple phosphorylations regulate the rapid degradation of Wee1 at M - phase (Watanabe *et al.*, 1995). First, it is a direct phosphorylation target of CDK1 itself that phosphorylates Wee1 on serine 123 (Watanabe *et al.*, 2004). Second, the serine 123 phosphorylation enables CK2 to phosphorylate it on another serine residue, serine 121. Third, CDK1 - CK2 double phosphorylated Wee1 is subsequently a suitable target for polo - like kinase 1 (Plk1) that phosphorylates serine 53 residue of Wee1 (Watanabe *et al.*, 2005). After these three phosphorylation events, Wee1 is efficiently recognized by SCF - β - TrCP ubiquitin ligase complex and directed it into proteosomal degradation (Watanabe *et al.*, 2004; Watanabe *et al.*, 2005). Further, it has been reported that AKT is able to phosphorylate Wee1 on serine

642, induce Wee1 binding to 14 - 3 - 3θ and cause it to translocate into cytoplasm. Thus AKT could promote G2/M progression (Katayama *et al.*, 2005).

Both Wee1 and CDC25 are phosphorylated by CHK1 after DNA damage, and this activates the cell cycle checkpoint. CDC25 phosphorylation by CHK1 is thought to promote its interaction with 14 - 3 - 3 proteins, which leads to the retention of the complex in the cytoplasm. At the same time, DNA damage causes the transient hyperphosphorylation and stabilization of Wee1A (Raleigh and O'Connell, 2000). As CHK1 and Wee1 regulate the G2/M checkpoint that in many cancer cells is the major remaining cell cycle checkpoint, their inhibition has been considered a potential target for cancer therapy (Dent *et al.*, 2011). Small molecule inhibitors of Wee1 have been reported to be efficient in combination treatment with IR and DNA – damaging agents in several cancer cell lines, including prostate cancer cell lines (Hirai *et al.*, 2009; Hirai *et al.*, 2010; Bridges *et al.* 2011). There are some indications that downregulation of Wee1 can abrogate G2 checkpoint and induce apoptosis in HeLa and breast cancer cells (Murrow *et al.*, 2010; Wang *et al.*, 2004).

2.4. DNA damaging agents as cancer therapy

Classical anticancer drugs can be divided into three categories: chemotherapy and IR, hormonal therapy and immunotherapy (Espinosa *et al.*, 2003). Chemotherapeutic drugs can further divided into subgroups based on their chemical structure and mechanism of action. Most of them cause excessive DNA damage, activate cell cycle arrest and cell death either directly or after DNA replication, and thus are efficient in killing proliferating cancer cells (Helleday *et al.*, 2008).

2.4.1. Cancer treatment by radiotherapy

Radiotherapy has long been used as a successful means of therapy in many cancer types, including prostate cancer (Bentzen, 2006). Its primary target is DNA. IR causes direct damage to the bases and the sugar - phosphate backbone (Hagen, 1994), and it is able to induce high number of DSBs, single - strand breaks and oxidative damage even in non - proliferating cells (Ciccia and Elledge, 2010). DSBs are considered the most toxic of all DNA lesions. It has been estimated that 1 Gy of IR causes on average 30 – 40 DSBs in a living cell (Lobrich *et al.*, 1994). There is variation in how well patients respond to radiation therapy. This is understandable, as radiation induces gene expression of thousands of genes and in some there is considerable variance between individual patients (Smirnov *et al.*, 2009).

2.4.2. Topoisomerase I and II inhibitors

DNA topoisomerases are enzymes that resolve topological problems that are caused by DNA unwinding (Nitiss, 2009a). When DNA molecule is opened up to allow transcription or replication, local unwinding causes supercoiling elsewhere. Topoisomerase enzymes cause spatially and temporally controlled breaks into DNA to relieve this stress. They are divided into two types by their mode of action: topoisomerase I enzymes (TOP1) introduce single strand breaks and topoisomerase II (TOP2) induce DSBs (Champoux, 2001).

TOP1 - inhibitors such as camptothecin (CPT) target human TOP1 enzyme (Liu *et al.*, 2000). CPT blocks reversibly the rejoining step in the cleavage - religation reaction and results in accumulation of the cleavage - complex on DNA. Combined with DNA replication this complex disturbs replication fork progression and causes cytotoxicity and DSBs in the S - phase (Furuta *et al.*, 2003; Kurose *et al.*, 2005). In addition, CPT may be cytotoxic outside of S - phase. CPT treatment prevents RNA synthesis by inhibiting transcription elongation, and can induce single - strand breaks and a small number of DSBs. It has been shown that CPT treatment results in DSBs and activation of ATM - pathway in post - mitotic neurons and lymphocytes via inhibition of transcription and formation of RNA - DNA - hybrids between open DNA and nascent RNA - transcript (Sordet *et al.*, 2009). It is noteworthy that this induction of DSBs and ATM activation in post - mitotic cells required considerable drug concentrations (Sordet *et al.*, 2009).

There are two types of TOP2 enzymes in mammalian cells: TOP2α and TOP2β (Nitiss, 2009a). TOP2α is essential for separating replicated chromosomes in all cells, while TOP2β is dispensable in some cell types. TOP2 - enzymes work as homodimers, where each subunit breaks one DNA strand. The enzyme is able to carry out the cutting without losing the energy of the phosphodiester bond in DNA, so that resealing of the cleavage does not require high - energy cofactors. Interestingly, TOP2β - mediated DSBs have been shown to be required for activation of gene transcription by 17β - estradiol - stimulated estrogen receptor (Ju *et al.*, 2006) as well as AR (Haffner *et al.*, 2010).

TOP2 - inhibitors such as doxorubicin and etoposide induce DSBs by trapping the TOP2 - enzyme in the DNA cleaving complex (Nitiss, 2009b). When TOP2 - inhibition is caused by the covalent trapping of the enzyme onto DNA, as is the case with doxorubicin and etoposide, the drugs are termed TOP2 poisons. TOP2 poisons do not require DNA replication to induce DSBs like some other drugs, e.g. cisplatin (Frankenberg - Schwager *et al.*, 2005). Doxorubicin may trap TOP2 by its intercalation into DNA - molecule. Etoposide differs from doxorubicin by the fact that it does not intercalate into DNA. Its effect is supposedly mediated by protein - DNA - interactions (Nitiss, 2009b). As TOP2 enzyme activity is essential for DSB induction by TOP2 - inhibitors, it is not surprising that resistance to TOP2 - inhibitors such as doxorubicin has been associated with a decrease in the protein expression levels (Burgess *et al.*, 2008).

2.5. DNA damage response in primary and terminally differentiated cells

The groundwork on DDR has largely been carried out in cancer cell lines, but there are a few reports investigating how various primary cells of mouse or human origin respond to IR. Especially mouse ES cells, MEFs, thymocytes and leukocytes have been used in many prominent studies and have been sited elsewhere in the text. Some key studies on radiation responses of various primary cell types are listed in **Table 2**.

p53 activation, apoptosis and cell cycle checkpoints have been studied in primary mouse cells and irradiated mice. p53 mediated apoptosis after IR was first characterized in mouse thymocytes (Clarke *et al.*, 1993; Lowe *et al.*, 1993), and IR induced G1 arrest in MEFs (Kastan *et al.*, 1992). Subsequent whole mice irradiation studies have clearly demonstrated that cell types differ in their radiation responses. For example, both epidermal and hair follicular cells of mouse skin stabilize p53, but epidermal cells respond with p21 - mediated growth arrest while hair follicular cells undergo apoptosis (Song and Lambert, 1999). The cellular outcome after DNA damage does not only depend on the cell type but also on the type of the damaging agent. For instance, p53 in mouse primary hepatocytes is stabilized after UV but not IR (Bellamy *et al.*, 1997), and doxorubicin and IR, but not cisplatin are able to activate p53 dependent G1 arrest in MEFs (Attardi *et al.*, 2004).

There are some reports on how human primary cells respond to IR. One of the first observations was that normal bone marrow progenitor cells induce p53 at low levels and are G1 proficient (Kastan *et al.*, 1991), which was followed by the notion that IR induces p53 dependent G1 arrest in normal human fibroblasts derived from foreskin or embryonic lung or skin (Di Leonardo *et al.*, 1994; Kuerbitz *et al.*, 1992). Then it was reported that, surprisingly, normal human prostate epithelial cells (HPECs) are unable to stabilize p53, p21 or activate G1 checkpoint while prostate stromal cells exhibit both responses (Girinsky *et al.*, 1995). Similar results were described using bronchial epithelial cells and lung fibroblast (Gadbois and Lehnert, 1997) and human keratinocytes (Flatt *et al.*, 1998). Further, when human mammary epithelial cells (HMECs) were reported to lack p53 and p21 stabilization and G1 arrest after IR (Meyer *et al.*, 1999), it seemed that the lack of p53 - mediated G1 arrest was a common theme of human epithelial cells. It is noteworthy that cell culture environment may promote growth of only one subtype of epithelial cells, as discussed in the chapter on prostate tumorigenesis, and this may have influenced the DDR studies carried out in primary cells. When HMECs were first sorted into basal and luminal cells using specific markers, and then cultured separately in optimized media, it turned out that IR can induce p53 and p21 transiently in basal cells without a strong cell cycle checkpoint activation (Huper and Marks, 2007). More interestingly, luminal cells were able to induce p53 and p21 in a more prolonged manner, arrested S - phase entry and accumulated in G2/M.

Table 2. *Radiation responses of benign primary cells.*

Cell	Tissue of origin	Species	Dose	p53	Response	Reference
Bronchial epithelial cells	Lung	Human	2 Gy	p53 -	Transient G1 and G2	Gadbois *et al.*, 1997
Bone marrow progenitor cells	Bone marrow	Human	~ 0 - 4 Gy	+	G1 and G2 arrest	Kastan *et al.*, 1991
Fibroblasts	Connective tissue	Human	0 - 6 Gy	+	G1 arrest	DiLeonardo *et al.*, 1994
HMEC	Breast	Human	4 Gy	p53 -	No G1, G2 arrest +	Meyer *et al.*, 1999
HMEC Basal	Breast	Human	5 Gy	p53 + 2 h	Transient G2	Huper *et al.*, 2007
HMEC Luminal	Breast	Human	5 Gy	p53 + 2 - 24 h	Prolonged G2 arrest	Huper *et al.*, 2007
HPECs	Prostate	Human	6 Gy	p53 -	No G1	Girinsky *et al.*, 1995
Keratinocytes	Skin	Human	8 Gy	p53 +/-	No G1, G2 arrest +	Flatt *et al.*, 1998
Hepatocytes	Liver	Mouse	15 Gy UVC 10 J/m^2	p53 – p53 +	Transactivation – Transact. +	Bellamy *et al.*, 1997
MEFs	Connective tissue	Mouse	5 Gy	p53 +	G1 and G2 arrest	Attardi *et al.*, 2004
Thymocytes	Thymus	Mouse	0 - 20 Gy	p53 +	Apoptosis	Lowe *et al.*, 1993, Clarke *et al.*, 1993

Terminal differentiation of some cell types can be induced in cell culture environment, and these models have been used to study DDR (listed in **Table 3.**). ATM - pathway, and to some extent p53 are activated in terminally differentiated, postmitotic cells of different origins after DSBs. First, neocarzinostatin (radiomimetic drug) treatment has been shown to activate ATM - pathway in human neurons (Biton *et al.*, 2007). Human embryonic stem cells and human neural stem cells from cerebral cortex were allowed to differentiate into mature neurons and were treated with neocarzinostatin. The induction of ATMS1981,

phosphorylation of serine 824 of KAP1, γH2AX, serine 15 phosphorylation of p53 and phosphorylation of threonine 68 of Chk2 in these cells clearly indicated that the ATM – mediated DSB response is activated in postmitotic cells. The response was shown to be primarily ATM - dependent, as concomitant use of ATM - inhibitor KU - 55933 or ATM depletion via shRNA abrogated the phosphorylation of the target proteins (Biton *et al.*, 2007). Second, ATM autophosphorylation, γH2AX induction, recruitment of Mre11 and Nbs1 and CHK2 activation have been shown to occur equally after IR in proliferating myoblast and terminally differentiated myotubes (Latella *et al.*, 2004). Further, p53 was not phosphorylated on serine 15, stabilized or transcriptionally active in myotubes after IR, while doxorubicin treatment did activate p53 and apoptosis in them (Latella *et al.*, 2004). Third, it is possible that terminal differentiation enhances some forms of DNA repair. In cell culture, mouse adipocytes were shown to repair DSBs more rapidly than preadipocytes, and this was associated with increased levels of DNA - PKcs activity (Meulle *et al.*, 2008). Inhibition of DNA - PK decreased repair efficiency only in differentiated apipocytes (Meulle *et al.*, 2008). Fourth, sometimes it is possible to analyze terminally differentiated cells directly out of patients, without long - term culturing artefacts. Löbrich *et al.* collected lymphocytes of 23 individual patients undergoing computed tomography (CT) examination of thorax and/or abdomen and studied formation and repair of DSBs after low doses of IR (ca. 100 – 1500 mGy*cm, roughly equivalent to 20 mGy) (Lobrich *et al.*, 2005). They investigated the γH2AX - levels of isolated lymphocytes either directly after the radiodiagnostic imaging or after a period of *in vitro* – culturing, and could show that this kind of an approach could be used to study DSB induction and repair *in vivo* as well as aid in optimizing radiation therapy doses and in identification of radiosensitive, repair - compromised patients.

Table 3. *DNA damage response in terminally differentiated cells.*

Cell	Tissue of Origin	Species	Dose	p53	DDR response	Reference
Adipocytes	Adipose tissue	Human/ Mouse	IR 2 Gy CLγ1	-	Enhanced NHEJ, DNA - PKcs +	Meulle *et al.*, 2008
Lymphocytes	Blood	Human	~20 mGy	-	γH2AX +	Löbrich *et al.*, 2007
Myoblasts	Muscle	Human/ Mouse	IR 10 Gy	p53 + Ser15 +	ATM - pathway +	Latella *et al.*, 2004
Myotubes	Muscle	Human/ Mouse	IR 10 Gy	p53 – Ser15 -	ATM - pathway +	Latella *et al.*, 2004
Neurons	Embryonic	Human	NCS	p53-	ATM -	Biton *et al.*,

	stem cells		100 - 200 ng/ml	Ser15 +	pathway +	2007
Neurons	Neural stem cells	Human	NCS 100 - 200 ng/ml	-	ATM pathway +	- Biton *et al.*, 2007

3. Organotypic Models in Cancer Biology

Different approaches have been used to set up new models to study various aspects of tumorigenesis and DDR in human tissues without disruption of the original tissue structure. These organotypic models have several advantages to standard cell culture techniques, as they enable the study of differentiated cell types that can be difficult or impossible to maintain in standard cell culture, conserve cell - cell contacts and heterotopic interactions between them and the supporting extracellular matrix that have been shown to modulate cell responses to stress. In addition, as practical mice models are, there are some limitations. First, there are considerable anatomical differences between mice and men in some organs. For example, mouse prostate has four paired, elongated lobes without a surrounding capsule, which does not entirely correspond to the human globular gland (Pienta *et al.*, 2008). Second, most transgenic prostate cancer mice models develop only hyperplasia and PIN or demonstrate rapid progression into poorly differentiated cancer. Third, due to the species difference, mouse models are not optimal for preventive or therapeutic discoveries (Pienta *et al.*, 2008).

3.1. Set up of organotypic models

Isolated cells can be directed into forming tissue like structures. Epithelial tissue stem cells are able to differentiate into multiple cell types and when implanted into mice together with supporting stromal cells, they can reconstitute the tissue structure. 3D culture systems are usually based on culturing of epithelial cells on Matrigel or other collagenous support that mimics extracellular matrix. This results in small glandlike structures that contain cells expressing several different cell type markers (Debnath *et al.*, 2003). A recent report indicated that normal primary human epithelial cells of epidermis, oropharynx, esophagus and cervix can be transferred onto a section of devitalized human acellular dermis complemented with stromal fibroblast, and they form a stratified, differentiated epithelium and regenerated stromal architecture. Use of transformed epithelial cells expressing oncogenic RAS or CDK4 resulted in invasion through basement membrane. This 3D

organotypic neoplasia model was suitable for testing of several small molecule inhibitors of signaling pathways, including mitogen - activated protein kinase (MAPK) and PI3K, as well as gene expression studies (Ridky *et al.*, 2010).

Ex vivo – tissue culture models are based on culturing of thin slices of tissue in an optimized medium in culture dishes outside of human/rodent body. The method has been used since 1920s, especially in studies investigating metabolism of tissues and neurophysiology and –pharmacology. An important technical advance was published in 1980, when Krumdieck et al described their new instrument for the preparation of tissue slices (Krumdieck *et al.*, 1980). The Krumdieck tissue slicer caused little damage to tissue architecture and had been used to prepare slices of rat liver, brain, spleen, kidney, heart, skin and adipose tissue (Krumdieck *et al.*, 1980). Since then, similar set - up has been confirmed to be feasible for various benign human and rodent tissues and tumor types, including human brain (temporal lobe cortex and white matter) (Nitsch *et al.*, 2000), rat enterorhinal - hippocampal brain sections and human glioblastoma tumors (Merz *et al.*, 2010), tumorous and non - tumorous human liver (Kern *et al.*, 2006), tumor xenografts of human ovarian cancer cell lines, human primary ovarian tumors and human liver (Kirby *et al.*, 2004), human fallopian tube epithelium (Levanon *et al.*, 2010), human colon, lung and prostate carcinomas (Vaira *et al.*, 2010), breast cancer (van der Kuip *et al.*, 2006) and rat small intestine and colon (de Kanter *et al.*, 2005).

Direct culturing of tissue fragments is possible, if the tissue consistency does not allow more precise slicing. At least fragments of human cervical tissue (Collins *et al.*, 2000), human testis (Roulet *et al.*, 2006) and human breast (Huper and Marks, 2007) have been used. If a more prolonged tissue culture time is required, the *ex vivo* - tissue slices or tissue fragments can be implanted subcutaneously or under the renal capsule of immunocompromised mice, which supports growth of vasculature.

3.2. Prostate organotypic models

Of the different approaches presented above, most have been set up of prostate tissue. Human embryonic stem cells grafted together with mouse urogenital sinus mesenchyme or rat seminal vesicle mesenchyme under the renal capsule of adult severe combined immunodeficiency (SCID) mice has been shown to mature into benign human prostate tissue, that expresses markers for basal (p63, high - molecular weight cytokeratins), luminal (AR and PSA), neuroendocrine (chromogranin A) and stromal cells (α - smooth muscle actin, α - SMA) within 8 - 12 weeks (Taylor *et al.*, 2006). Similarly, primary human prostate cancer cells isolated from Gleason grade 8 to 9 prostate cancer and further immortalized with transduction of exogenous telomerase have been shown to reconstitute the original tumor when combined with rat embryonic mesenchyme and grafted under the renal capsule of male SCID mice (Gu *et al.*, 2007). Furthermore, it has been shown that

with careful selection of prostate stem cell markers, it is possible to generate functional prostate tissue from a single adult murine prostate stem cell (Leong *et al.*, 2008; Wang *et al.*, 2009)

Three - dimensional culture models of prostate tissue have been successfully established. Human prostate epithelial cells immortalized with telomerase expression form spheroids on Matrigel and differentiate into basal and luminal cells (Kogan *et al.*, 2006). SV40 T antigen - immortalized human prostatic epithelial cells (BPH - 1) grown on Matrigel have been shown to form prostatospheres that mimic the polarized glandular structure of prostate, as did their tumorigenic subline (Chu *et al.*, 2009). This 3D - model was then successfully applied to studying the role of androgen signaling and PI3K - pathway in epithelial - mesenchymal transition. On the other hand, HPV16 E6/E7 –immortalized or human telomerase reverse transcriptase –immortalized, non - tumorigenic prostatic epithelial cell lines did not undergo similar differentiation process under the same circumstances (Chu *et al.*, 2009).

Ex vivo – tissue culture models of prostate have been shown to be applicable to short - term experimentation. Bläuer et al cultured benign prostate slices of 300 μm in a serum and growth factor free medium supplemented with DHT for seven days, and based on cell - type specific markers cytokeratin 18 and 14, AR, PSA, prostate acid phosphatase (PAP) and von Willebrand factor (endothelial cell marker) showed that major cell types remain viable (Blauer *et al.*, 2008). It is possible to combine the benefits of *ex vivo* – tissue culture and xenografting. Subcutaneous implantation of small (2 - 3 mm x 1 - 2 mm) pieces of benign prostate or prostate cancer into the flanks of athymic nude mice has been reported to extend the viability time of the tissue pieces into 1 month (Gray *et al.*, 2004). Implantation of thin slices of benign human prostate tissue or primary adenocarcinomas under the renal capsule of immunodeficient mice was reported to enable the slices to remain viable and to display characteristic histology and expression of cell - type specific markers for at least three months (Zhao *et al.*, 2010). A considerable amount of the vasculature of the grafts was lined with human endothelial cells in this model (Zhao *et al.*, 2010).

In the stem cell based models, prostate tissue is derived from a limited number of human prostate cells, so it is possible that genetic changes acquired early in the development process in mice have enriched into the tissue, and although the microenvironment under the renal capsules of immunocompromised mice supports the growth and viability of human prostatic tissue, it may not fully recapitulate all the interactions of the surrounding tissue environment of human prostate. In these xenograft models prostate tissue can be maintained for several months and are suited for studying the effects of vasculature and immunosystem.

3.3. DNA damage response in human organotypic models

Most studies investigating DDR have used cancer cell lines, mouse models and standard immunohistochemistry (IHC) techniques, but some work has been carried out in human organotypic models.

Huper *et al.* (2007) used human breast tissue to study p53 induction after IR. They cultured breast tissue fragments of one patient overnight, treated them with 5 Gy of IR and incubated for 2 or 24 hours. The fragments were then frozen, sectioned and analyzed with IHC. The histology of the tissue fragments appeared normal and apoptosis was low. Two hours after IR, p53 induction was evident only in basal cells, but 24 hours after IR it was primarily detected in the luminal compartment. Similarly, H2AX phosphorylation was reportedly removed more rapidly from the basal cell compartment, while luminal cells expressed a more prolonged response (Huper and Marks, 2007). Another, a more extensive study on human breast tissue fragments used mouse xenografting, and analyzed the p53 pathway activation and γH2AX responses of 10 patients four days after implantation (Coates *et al.*, 2010). The percentage of apoptotic cells increased from 0,14 % to 4 % already four hours after 5 Gy. p53 was stabilized and phosphorylated on serines 15 and 392 in breast epithelium in a dose dependent manner after IR, while the stromal responses were minimal. p21 stabilization was more pronounced in basal cells. Surprisingly, γH2AX foci were detected only in the epithelial cells, and they were stronger in epithelium surrounded by adipocyte - rich stroma than by fibroblast - rich stroma (Coates *et al.*, 2010).

Levanon *et al.* (2010) set up two new *ex vivo* - models of human fallopian tube epithelium and used them to study early DDR activation after IR treatment in the two epithelial cell types of fallopian tubes, ciliated and secretory cells. First, they collected benign fallopian tube specimens from surgical procedures, minced and digested with protease and DNase treatments and cultured the cell isolates on the air - liquid interface of polyester membrane. These cultures were shown to contain both cell types and could be maintained for several weeks, and interestingly, when these *ex vivo* – cell cultures were treated with IR, $ATM^{Ser1981}$ phosphorylation and γH2AX inductions were more pronounced in the secretory cells that had proliferative capacity. Second, they used fresh, whole fallopian tube fragments to confirm that induction of γH2AX, CHK2 and $ATM^{Ser1981}$ phosphorylation and stabilization of p53 were more pronounced in the secretory cells than in the terminally differentiated, quiescent ciliated epithelial cells (Levanon *et al.*, 2010).

Aims of the study

Eukaryotic cells have evolved multiple pathways to detect and repair various forms of DNA damage to protect their genomes from accumulating genetic aberrations, the driving force in tumorigenesis. Characterization of these DDR pathways has successfully been carried out in cancer cell lines and mouse models, but they do not take into account the effects of cell differentiation and signaling from other cell types and extracellular matrix present in adult human tissues.

This study was aimed at studying how normal human epithelial cells of two organs of the male reproductive system, prostate and seminal vesicle, recognize and respond to exogenous DNA damage during active replication and in quiescent, terminally differentiated state, and to look for factors that could explain the major difference in their susceptibility to tumorigenesis.

The specific aims were to:

1. investigate which DNA damage recognition pathways are activated by various forms of genotoxic and cellular stress factors in benign human prostate tissue
2. study whether the three main cell types of adult human prostate differ in their DNA damage responses
3. determine the cell cycle checkpoint proficiency of human primary epithelial cells
4. to compare DNA damage responses in prostate and seminal vesicles

Materials and methods

The materials and methods used in the study have been described in detail in the original publications (I - III). A list and a short description of the most relevant materials and methods are provided below.

1. Materials

1.1. Patient material (I, II, III)

Patient material used in this study includes *ex vivo* – cultured live tissue samples and isolated primary epithelial cells of prostate and seminal vesicle, as well as standard paraffin embedded tissue material. *Ex vivo* - and primary cell material was collected from patients undergoing radical prostatectomy or cystectomy due to prostate or bladder cancer with informed consent of the patients and the permission of the Ethics Committee, Department of Surgery of Helsinki University Central Hospital.

1.2. Antibodies (I, II, III)

All primary antibodies were purchased from commercial sources listed below (**Table 4.**). The fluorescent Alexa secondary antibodies were ordered from Molecular Probes and the biotinylated or horseradish peroxidase – conjugated secondary antibodies from Dako.

Table 4. *Antibodies*

Antigen	**Clone**	**Manufacturer**	**Used in**
5 – Bromo – deoxy - uridine		Sigma-Aldrich Amersham Pharmacia	I II
53BP1		Novus Biologicals	I, III
α - SMA		Sigma-Aldrich	I, III
β - tubulin		BD Biosciences	II
Phosphorylated AKT		Cell Signaling	Unpublished
Androgen receptor		Biocare Medical	I, III
$ATM^{Ser1981}$		Cell Signaling	I, III
Cdc25A	F - 6	Santa Cruz	II
Cdk2	M2	Santa Cruz	II

Cdk1/2 - Tyr^{15}		Cell Signaling	II
		Calbiochem	II
Cleaved caspase - 3	5A1E	Cell Signaling	III
Cytokeratin 5 and 14		Imgenex	I, III
ERG		Epitomics	III
GAPDH		Europa Bioproducts	I, II, III
H2AX		Novus Biologicals	I, III
		Cell Signaling	I, III
γH2AX	Clone JBW301	Upstate Biotecnology	I, II, III
	20E3	Cell Signaling	I,
	pSer^{139}	Epitomics	I, III
Ki67	MIB - 1	Dako	I, III
Mucin - 6	MUC - 6	Novocastra	III
p21	12D1	Cell Signaling	I, III
	C - 19	Santa Cruz	II
	6B6	BD Biosciences	II
p27	p27/Kip1	BD Biosciences	I
		Epitomics	I
		Santa Cruz	II
p53	DO - 7	Dako	I, II, III
	7F5	Cell Signaling	I,
	DO - 1		II, III
p63	Ab - 4	Neomarkers	I, II, III
PSA	A0562	Dako	I
TOP2B		Atlas Antibodies	III
Ubiquitin	FK2	Enzo Life Sciences	III
Wee1A	H - 300	Santa Cruz	II, III
	B - 11	Santa Cruz	III

1.3. Cell lines (I, II, III)

Established cancer cell lines, immortalized human fibroblasts and human keratinocytes used in these studies were commercially derived or kindly provided by our collaborators **(Table 5.)**. The primary prostate and seminal vesicle epithelial cells were isolated from the patient samples. The tissue sections were cut into small pieces of less than a 1 mm3, placed onto collagen IV –coated petri dishes in an optimized culture medium and kept in culture until an epithelial cell population was established. The cells were then collected and stored in – 130 °C until further propagated for up to four passages.

Table 5. *Cell lines*

Cell line	Description	Source	Used in
A375	Human malignant melanoma	ATCC	II, III

HeLa	Cervical cancer cell line	ATCC	III
HK	Human keratinocytes	Promocell (NHEK)	III
HPEC	Human prostate epithelial cells	Universities of Stanford and Helsinki	II, III
HSVEC	Human seminal vesicle epithelial cells	University of Helsinki	III
LNCaP	Metastatic prostate cancer cell line	ATCC/Donna Peehl	I, III
MCF10A	Human mammary epithelial cells	ATCC	II
Mv1Lu	Mink lung epithelial cells	ATCC	II
PC3	Prostate cancer cell line	ATCC/Donna Peehl	III
U2OS	Osteosarcoma, p53 wt	ATCC	I, II, III
WS-1	Human skin fibroblast	ATCC	II, III

2. Methods

Method	**Used in**
5-BrdU labeling	I
Cell culture	I, II, III
Confocal microscopy	I, III
Ex vivo – tissue culture	I, II, III
Fluorescent activated cell sorting (FACS)	II, III
Gene silencing via RNAi	III
Image analysis	I, III
Immunofuorescence	I, II, III
Immunohistochemistry (IHC)	I, II, III
Transfection of cells	III
Transduction of cells	III
TUNEL labeling	III
Western blotting	I, II, III

2.1. *Ex vivo* – tissue culture and DNA damaging treatments (I, II, III)

Ex vivo – tissue culture method utilizes thin, precision cut tissue slices that can be maintained viable for a limited period of time. A cylinder of 8 mm in diameter and several centimeters in length was cored fresh from the prostate and seminal vesicle tissue derived from surgery. The cylinder was then cut into 300 – 500 μm slices with Krumdieck Tissue

Slicer, Alabama Research and Development and transferred onto titanium grids. The slices were maintained under rotation and in an optimized culture medium in - 37°C and 5 % CO2 for 1 - 7 days. R1881 androgen analogue (10 – 20 nmol/l) was added to the culture medium if not otherwise indicated.

The tissue sections were treated with 10 Gy of IR using a calibrated 137Cs γ - source (Biomeam 800, STS). Doxorubicin, etoposide, camptothecin, cisplatin, actinomycin D and MG132 proteasome inhibitor were all purchases from Sigma - Aldrich, diluted into DMSO or ethanol and added to the culture medium at appropriate concentrations. The sections were fixed with 10 % formalin, Oy FF Chemicals Ab, after the indicated incubation times of 2 – 24 hours.

2.2. Immunohistochemistry (IHC) (I, II, III)

The paraffin embedded tissue sections were cut into 4 µm sections and stored in + 4 °C. The sections were deparaffinized, rehydrated and the antigens retrieved with heat induced - epitope antigen retrieval by heating the slices in standard acidic sodium citrate or basic Tris - EDTA buffers in a microwave oven (700 – 800 W) for up to 30 minutes. The unspecific peroxidase activity was quenched in 0,5 % - 3 % H2O2 –solution, and unspecific background signal blocked with 2 - 5 % normal goat serum, Dako and 5 % milk. The primary antibodies were diluted into the blocking buffer and incubated for 1h in room temperature or overnight in +4°C. The biotinylated secondary antibodies were diluted 1:100 and incubated for 30 minutes. The ABC Vectastain – Kit was used to enhance the signal detected with AEC or DAB+, Dako. The sections were counterstained with hematoxylin, mounted and the signal visualized with light microscopy.

The immunofluorescent staining of tissue sections was carried out similarly to standard IHC protocol with the exception of omitting the quenching of unspecific peroxidase activity. The secondary antibodies were conjugated with the appropriate fluorescent labels and no further signal amplification was required. The counterstaining was carried out with Hoechst.

2.3. TUNEL labeling (III)

Terminal deoxynucleotidyl transferase – mediated dUTP nick end (TUNEL) - labeling was used to detect and estimate the number of apoptotic cells in fixed, paraffin embedded *ex vivo* – tissue sections. Terminal deoxynucleotidyl transferase enzyme catalyzes the polymerization of labelled nucleotides to the ends of DNA fragments created in apoptotic cell death. This allows rapid and sensitive detection of apoptotosis at a single cell level.

TUNEL - labeling was carried out with a commercially available In Situ Cell Death Detection Kit, TMR red by Roche Diagnostics, where the nucleotides are labelled with a red fluorescent marker and apoptotic cells can be directly visualized with fluorescence microscopy. The tissue sections were treated according to the manufacturer's recommendations for difficult tissue.

2.4. 5 - BrdU labeling (I, II, III)

5 - Bromo - deoxyuridine (5 - BrdU) labeling is used to detect cells in S - phase. 5 - BrdU is a thymidine analogue that can be incorporated into newly synthesized DNA and detected with antibodies. Cells and tissues were incubated with 50 – 100 μM 5 - BrdU for 30 min – 2 h, and subsequently fixed and stained for 5 - BrdU. In tissue samples the heat induced antigen retrieval is sufficient for exposing the epitope, but with cell culture samples an additional treatment with 1,5 M hydrochloric acid for 20 min prior to blocking of unspecific signal was required.

2.5. Cell culture, transfection of cells and gene silencing via RNAi (II, III)

All the cells were cultured in their appropriate culture media in + 37 °C and 5 % CO2 in a humified atmosphere. Cell transfections were carried out with lipofection using commercial reagents FuGENE 6 or FuGENE HD transfection reagents, Roche Diagnostics or Lipofectamine 2000, Invitrogen, according to the manufacturers recommendations, or by electroporation with Gene Pulser II instrument, Bio - Rad.

RNAi silencing of p53 was carried out with lentiviral transduction. To produce the viral particles, 293T cells were transfected with the lentiviral gag, pol, rev and VSV - G envelope protein - carrying genes and either pLKO.1 scrambled vector or shRNA targeting p53 in pLKO.1 (# TRCN0000010814 or # TRCN000003753, Open Biosystems). The viral supernatant was added to the primary cells with 8 mg/ml polybrene to enhance the infection, and the media was replaced after 8 hours. IR treatment of the cells was carried out on the third day after viral transduction.

2.6. Fluorescent activated cell sorting (FACS) (II, III)

FACS analysis allows cell sorting and cell cycle analysis based on cell size and detection of their DNA content. Briefly, cultured cells were collected and fixed with 70 % cold ethanol overnight. The cells were permeabilized with 0,1 % TritonX - 100 for 5 min, treated with 0,12 μg/μL RNAse to remove RNA and 0,12 μg/μL propidium iodide to stain the DNA. The cell cycle profile of the cells was analyzed with an LSR flow cytometer, BD

Biosciences, San Jose, CA and the analyzes performed by ModFit LT vs. 3.1, Verity Software House, Topsham, ME.

2.7. Immunofuorescence (I, II, III)

The cells were grown on coverslips and fixed with 3,5 % PFA in PBS for 20 min. Unspecific binding was blocked with 3 % BSA in PBS for 5 min, the primary antibodies diluted into 1 % BSA and incubated in +37 °C for 30 min – 1h. The secondary antibodies were diluted 1:200 and incubated in +37 °C for 30 min – 1h. DNA was counterstained with DAPI or Hoehcst, and the coverslips mounted with Vectashield, Vector Laboratories.

2.8. Western blotting (I, II, III)

Cells were collected, washed and lyzed with urea/Tris buffer (9 M urea/75 mM Tris - HCl, pH 7.5 supplemented with 0.15 M 2 - mercaptoethanol), and the pellet was sonicating briefly. The protein concentrations were determined by Bio - Rad assay and the proteins (50 - 100 mg for UTB lysates) were separated by SDS/PAGE and transferred to polyvinylidene fluoride membranes, Immobilon P, Millipore Corporation. Unspecific signal was blocked in 3 % BSA or 5 % milk, the primary antibodies diluted in 1 % BSA or 5 % milk. HRP - conjugated secondary antibodies were diluted in 1 % BSA, and the signal detected with enhanced chemiluminescence.

2.9. Confocal microscopy and image analysis (I, III)

Confocal images were acquired using Zeiss LSM 510 META confocal microscope, Plan - Neofluar 40x/1.3 Oil DIC and Plan - Apochromat 63x/1.4 Oil DIC objectives, LP420, BP 505 - 530 and LP 560 filters and LSM software release 3.2, Jena, Germany.

In order to decrease variability in IHC sample analysis, we developed a Matlab based image analysis application called Cell Image Segmentation and Classifyer (CISC). CISC is able to analyze multiple sets of images from tissue sections labelled with fluorescent antibodies. It detects nuclei stained with DAPI or Hoechst based on their fluorescent signal intensity, shape and size, and can thereafter be used to quantify nuclear fluorescent signal of 1 - 2 proteins detected with appropriate immunofluorescent labels. The detected cells can be divided into 2 – 6 different groups based on their mean signal intensity and distribution using K - means algorithm. For example, γH2AX signal was classified into four intensity groups that were determined as negative, low positive, high positive and apoptotic cells. Fluorescence images analyzed with CISC were acquired with Zeiss Axioplan 2 upright

epifluorescence microscope, Zeiss Plan - Neofluar objectives, Zeiss AxioCam HRm 14 - bit grayscale CCD camera and Zeiss Axiovision 4.6 software.

2.10. Tissue microarray analysis of Wee1A in prostate tumors (Unpublished)

Wee1A and p27 levels as well as phosphorylated AKT were determined using IHC from two sets of tissue microarrays (TMA) built by Tapio Visakorpi at the University of Tampere, and their association with known clinical prognostic markers was estimated. The primary prostate cancer TMA consisted of 248 prostate tumors, three cores of each tumor. The androgen - resistant prostate tumor TMA consisted of 173 cancer samples. The TMA slides were deparaffinized, rehydrated and antigens retrieved by heating the slides in 0.01 M sodium citrate (pH 6.0) or Tris - EDTA (pH 9.0) buffer (700 – 800 W, up to 20 min). The following antibodies and dilutions were used: Wee1A mouse monoclonal Ab (B - 11, Santa Cruz Technologies) 1:100, phosphorylated ATK (Cell Signaling) 1:50 and p27 (C - 19, Santa Cruz) 1:50. p27 and phosphorylated ATK were detected with Vectastain ABC - kit, and Wee1A with ADVANCE kit obtained from Dako according to the manufacturers instructions.

The Wee1A staining was inspected blindly by two independent observers and scored into the following groups in the primary prostate cancer TMA: negative (165 cases), low – moderate nuclear expression (36), high nuclear expression (8), high cytoplasmic localization (21) and non - readable (28). In the androgen resistant tumors Wee1A expression was classified as negative (71 cases), nuclear expression (5), cytoplasmic expression (13), nuclear Wee1 expression combined with homogenous cytoplasmic expression (5), nuclear Wee1A expression and strong cytoplasmic localization in a fraction of the cells (6) and non - readable (73).

Statistical analysis was carried out by using Kruskal - Wallis test, chi - square test and Mann - Whitney U - test.

Results and discussion

1. *Ex vivo* - tissue culture

Studying the early steps in carcinogenesis by using cancer cell lines has its challenges, and mouse reproductive organs have anatomical and physiological differences compared to human prostate and seminal vesicles. To overcome these limitations we employed *ex vivo* – tissue culture to study acute DDRs of two benign human tissues, prostate and seminal vesicles. We hoped to gain a better understanding of how normal human epithelium deals with DNA damage and to lay groundwork for deeper understanding of how genetic aberrations start to accumulate in prostate cancer.

1.1. Setting up the *ex vivo* – assays (I, III)

Ex vivo - tissue culture method is based on culturing of thin slices of prostate or seminal vesicle tissues for limited periods of time (I, Figure 1A, overview of the method). The tissues are collected with informed, written consent of patients undergoing prostatectomy due to underlying bladder or prostate cancer. One of the first principles of setting up this kind of assays is that patient diagnosis cannot be compromised. To ensure this in our work, apex and basis of the cored prostate tissue (8 mm in diameter) and a certain percentage (20 – 35 %) of collected tissue slices were designated for standard diagnostic evaluation. Benign prostate tissue samples were collected from peripheral zone apart from the tumor region detected in the needle biopsies, and seminal vesicle material from the superior end of the gland.

Prostate and seminal vesicle tissues were suitable for *ex vivo* – culturing for multiple reasons. As approximately 1000 radical prostatectomies are carried out in Finland every year, tissue material is readily available. The solid consistency of prostate and to some extent seminal vesicles enabled the precise slicing of the tissue. To support the viability of the tissue slices during culturing, the culture medium was optimized to contain the right amount of essential amino acids, vitamins, trace elements and growth factors. The survival of the prevalent cell types in *ex vivo* – slices was confirmed with detection of specific marker proteins (I, Figures 1, S2, S3 and III, Figure S2), and levels of replication and apoptosis were shown to be comparable to standard paraffin embedded tissue sections (I, Figures 1D, 2D and S5 and III, Figures S2 and S3).

There are certain limitations in using *ex vivo* – assays. First, as patient material was obtainable only from radical prostatectomies due to bladder or prostate cancer that mostly affect older men, it is possible that aging and age - related pathologies influenced the

studied biological responses. It is also possible that some traits that predispose men to tumorigenesis were enriched in our material, but excluding these caveats by patient selection is not ethically feasible. Second, from a technical standpoint, manipulation of tissue responses could only be carried out with radiation, drugs and small molecule compounds that have good tissue permeability. Direct modulation of protein expression by gene silencing or transfection of the *ex vivo* – slices is difficult and, to our knowledge, reports describing such approaches have not been published. Lentiviral transduction holds most promise in achieving this goal, which would make the *ex vivo* – culture model more versatile in studying basic biological mechanisms.

Due to PSA - screening and earlier detection of prostate cancer, tumor size at the time of diagnosis has decreased over time, but with careful patient selection it is also possible to collect *ex vivo* – material of prostate tumor regions. As a proof of principle, Vaira *et al.* (2010) published a similar organotypic assay with 213 tumor samples of 13 different tumor types including prostate cancer. In future, organotypic assays could be beneficial in optimizing the choice of drug treatment for individual cancer patients as well as validation of efficacy of novel therapeutic drugs in human tissue before clinical trials.

1.2. Development of image analysis tools for quantification of the DNA damage response (I)

IHC is a semiquantitative method. It allows the detection of moderate to major changes in protein expression level or localization. It relies on the specificity of the primary antibodies used, and sensitivity or detection limit depends on the method of signal amplification and visualization. Quantification of IHC results has traditionally been carried out by visual estimation of signal intensity and the percentage of positive cells. Visual quantification can be influenced by many factors, including personal experience of the surveyor, changes in cell density, unspecific background, staining and sample quality.

CISC has several advantages compared to visual quantification. There is always some autofluorescence and other unspecific signal in tissue samples, but the background levels of fluorescence can be estimated with appropriate negative controls. After acquisition of the images, the data analysis is fairly straightforward and rapid. The number of detected objects from one sample (2 - 5 fields/images) varies from several hundred cells to thousands, which is sufficient for further statistical analysis. As the same algorithm is used for all images in the dataset, there is no variance in parameters that define the assignment into groups. It is also possible to use same parameters for other analyses later on, that further minimizes the risk of a drift in quantification criteria. In general, CISC allows a more reliable quantification of a larger number of cells than what can be achieved with more conventional methods.

1.3. Early DNA double strand break recognition in prostate and seminal vesicle epithelium (I, II, III)

Considering how resistant prostate adenocarcinomas are to various forms of chemotherapeutic drugs (Mike *et al.*, 2006), it was unclear how well DNA damage could activate DDR in benign human prostate tissue. One of our hypotheses at the beginning of the study was that perhaps DNA damage was unable to activate essential DDR proteins already in normal tissue and that this could explain the lack of efficacy of chemotherapeutics in tumors. In addition, we were interested in whether there would be significant differences in the DDR of major cell types.

We focused our efforts on studying how early DNA damage recognition would be activated by DSBs that are relevant in formation of gene fusions detected in prostate cancer (Bartek *et al.*, 2010), and chose to use IR and topoisomerase 2 poisons as damaging agents. Based on the induction of $ATM^{Ser1981}$ and its accumulation into DNA damage foci, IR, doxorubicin and etoposide all readily activated the early DSB sensor ATM in the three major cell types of prostate (I, Figures 6A and S10) and seminal vesicle *ex vivo* – slices (III, Figure 1 and **Figure 7.**). This is in concordance with ATM being the major regulator of the DSB response (Bensimon *et al.*, 2010). It is essential for the repair of 10 – 25 % of DSBs (Goodarzi *et al.*, 2008), and defects in its function would render cells sensitive to irradiation (Barlow *et al.*, 1996; Rainey *et al.*, 2008; Xu and Baltimore, 1996). Neither prostate nor seminal vesicles are particularly radiosensitive. The downstream signal transducer 53BP1 accumulated into similar foci in all the cell types and at equal magnitude in both prostate and seminal vesicles (I Figure 6B, S11, S12, III Figures 1 and S4 and **Figure 7.**) indicating that ATM - pathway activation occurs rapidly and universally after DSBs in these tissues. The fact that 53BP1 foci are retained in G0/G1 and terminally differentiated cells supports the notion that 53BP1 has cell cycle independent functions, most likely in chromatin modification and promotion of NHEJ (Dimitrova *et al.*, 2008; Noon *et al.*, 2010; Xie *et al.*, 2007).

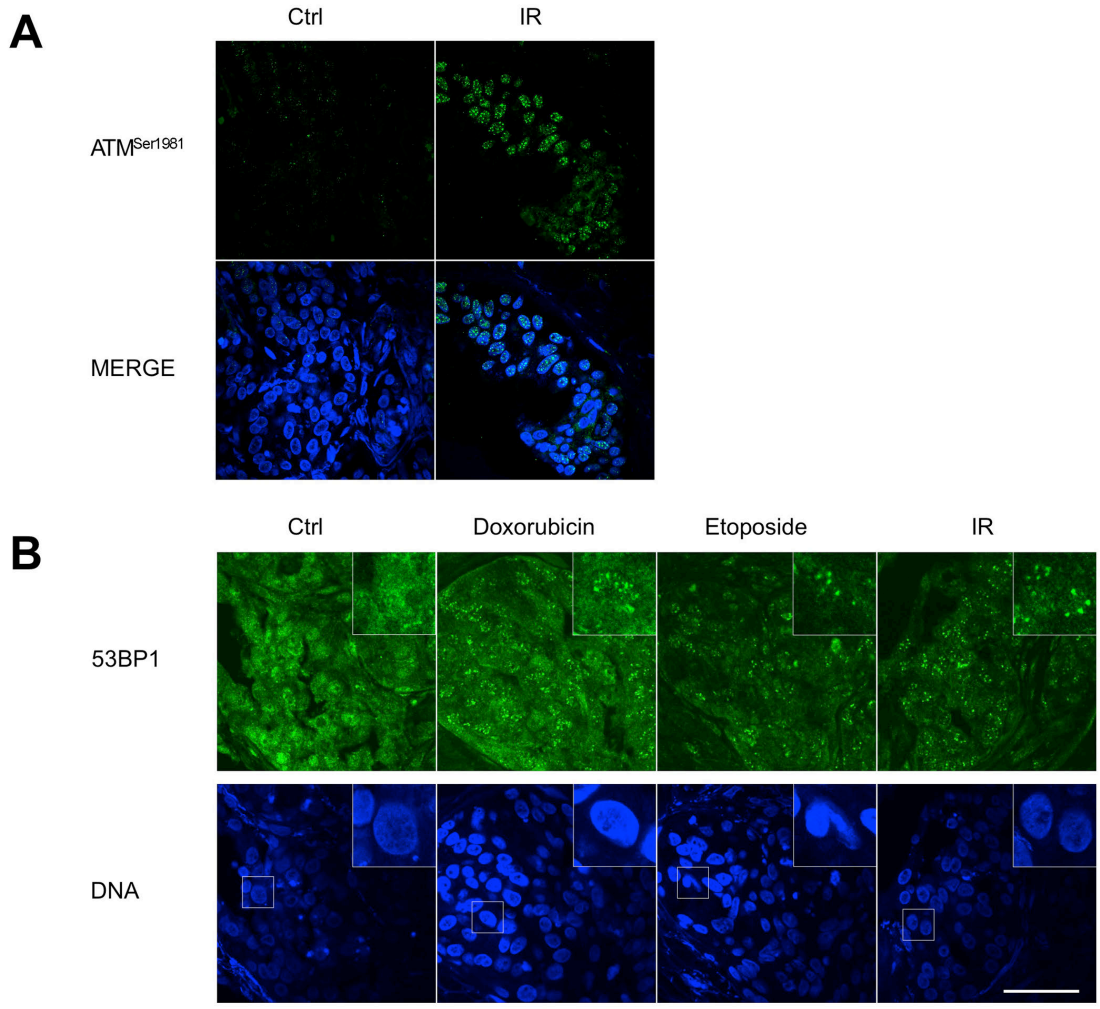


Figure 7. *ATM $^{Ser\ 1981}$(A) and 53BP1 expression (B) in seminal vesicles after DNA damaging treatments.*

1.3.1. The role of H2AX in replication proficient and terminally differentiated cells

Since their identification in 1998, γH2AX foci have been used as a sensitive marker for DSBs (Rogakou *et al.*, 1998). It has been noted that S - phase cells and some cancer cell lines have high endogenous levels of γH2AX and further, that other types of DNA damage combined with DNA replication may lead to γH2AX induction independent of DSBs. However, with careful planning of the experiments, they provide a reliable means to estimate the number of DSBs as well as DSB repair kinetics after IR (Lobrich *et al.*, 2010). In the prostate and seminal vesicle *ex vivo* – culture models, the number of replicating cells is low (I, Figure 1 and III, Figure S2D) and approximately 99 % of cells are in G0/G1. γH2AX detection is thus suitable for analysis of DSBs in both models, and co -

immunostaining of γH2AX with cell type specific markers enables comparison between different cell types.

As expected, treatment with IR and TOP2 - inhibitors doxorubicin and etoposide resulted in a robust induction of γH2AX foci in both prostate (I, Figures 2A, 2B and S6) and seminal vesicle tissue (III, Figure 2 and unpublished data) confirming that these agents are potent inducers of DSBs. In prostate tissue, there was a considerable decrease in the total levels of γH2AX from 8 hours after IR (more than a 25 - fold induction compared to non - treated slices) to 24 hours after IR (less than a 15 - fold induction), most likely due to DSB repair. γH2AX levels decreased also from 8 to 24 hours after doxorubicin and etoposide treatment, but as the drugs were not washed away from the culture medium, it is harder to specify whether this was due to DSB repair or some other adaptation response. In order to confirm that the γH2AX levels were not induced by apoptosis (Rogakou *et al.*, 2000), we carried out a co - immunostaining with cleaved caspase - 3, a known marker for apoptosis (I, Figure S5). The apoptotic γH2AX signal was pan - nuclear and highly intensive, and it could be separated in the image analysis. The percentage of apoptotic cells caused by the DNA damaging treatments of prostate *ex vivo* – slices ranged from almost zero to 10 %, and on average it was 5 %. In seminal vesicle tissue the apoptotic response was not quantified, but was confirmed to be low and comparable to prostate by TUNEL - assay (III, Figure S3).

γH2AX is considered essential for efficient DSB repair (Bonner *et al.*, 2008) and has been shown to protect genomic integrity in mouse models (Bassing *et al.*, 2002; Bassing *et al.*, 2003; Celeste *et al.*, 2003a). It was therefore rather unexpected that after DSBs induced by IR or TOP2 - inhibitors, postmitotic luminal cells of prostate tissue did not show as strong γH2AX signal as replication proficient basal or stromal cells (I, Figures 5A, 5B and S9). Considering that ATM activation occurred in all the cell types, the 10 times lower levels of γH2AX signal in luminal cells were not likely caused by disrupted ATM kinase function. We hypothesized that maybe the total level of histone variant H2AX itself is low in luminal cells, and could verify this with IHC of standard paraffin embedded prostate tissues (I, Figure 5C and 5D and **Figure 8.**). It is tempting to speculate that the lack of H2AX would render luminal cells more vulnerable to DSBs and genomic rearrangements, but technical limitations of *ex vivo* – tissue culture (they are not amenable to genetic manipulation or transfection, the number of mitotic cells is low and the culture time is limited) prevented a more formal proof of the concept. We then turned to the seminal vesicle *ex vivo* – model in search for indirect evidence, but surprisingly, secretory cells of seminal vesicle tissue responded in a similar manner. After IR, the levels of γH2AX were lower in secretory than in basal epithelial cells (III, Figure 2B). IHC of standard paraffin – embedded seminal vesicle tissue confirmed that compared to the basal cells, H2AX - levels of secretory seminal vesicles cells are low. Interestingly, some stromal cells of prostate and seminal vesicle tissues do not express the expected levels of H2AX (**Figure 8.**).

Furthermore, we noted that the maximal induction of γH2AX after IR treatment seemed to occur more rapidly and the resolution of the foci was slower in the seminal vesicle tissue (III, Figure 2). It is possible that this reflects differences in DSB repair kinetics between the two tissues, but confirmation of this hypothesis would require further studies.

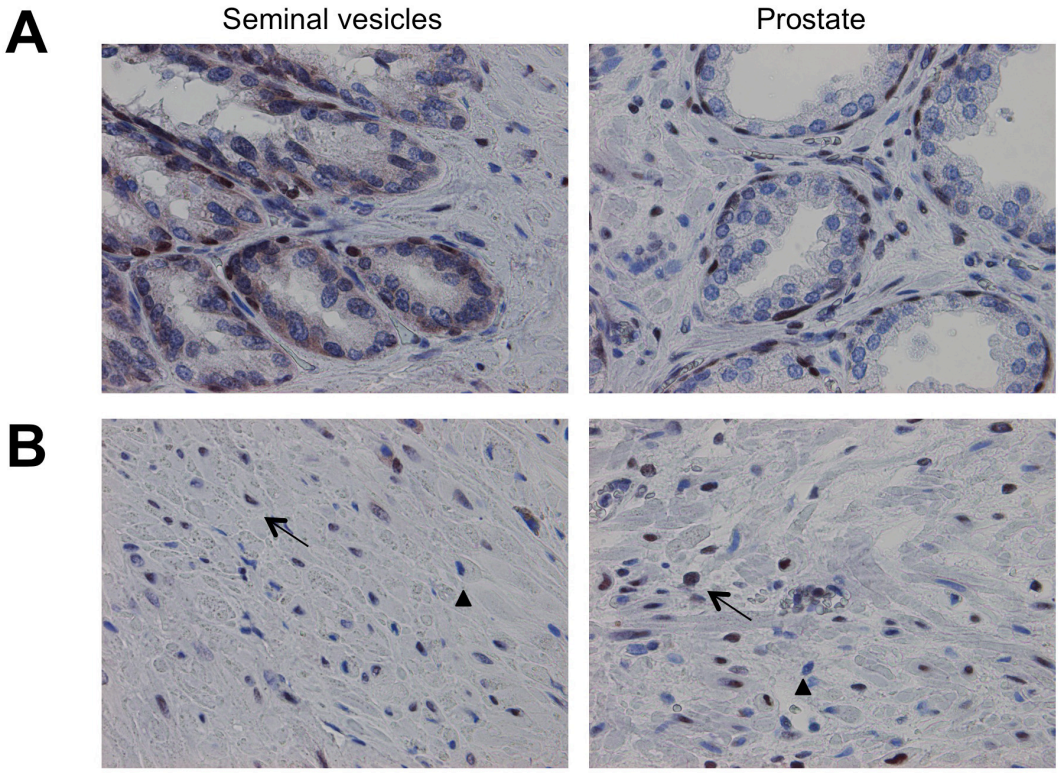


Figure 8. *H2AX expression in prostate and seminal vesicle epithelium (A) and stroma (B). Arrows indicate H2AX positive cells and arrowheads H2AX negative cells.*

H2AX can be expressed in quiescent cells independently of replication (Wu *et al.*, 1982). If maintenance of H2AX levels was indispensable in terminally differentiated cells, they should be able to produce more H2AX protein in order to compensate the inevitable wear and tear. The fact that secretory cells of both prostate and seminal vesicles do not retain H2AX at comparable levels to basal cells suggest that either high amounts of H2AX are not essential for DSB repair in terminally differentiated cells, or that they tolerate the reduction in efficient DSB repair caused by decreased γH2AX. The first possibility is supported by our observation that 53BP1 can still accumulate into DNA damage foci and be retained there for at least 24 hours after IR. γH2AX has been considered essential for recruitment and anchoring of other DSB signaling and repair factors onto damaged chromatin (Celeste *et al.*, 2003b; Paull *et al.*, 2000), but maybe this function can be carried out with the minimal amount of H2AX present in terminally differentiated cells. It is possible that the γH2AX signal amplification covering approximately one megabase around a DSB in proliferating cells (Rogakou *et al.*, 1999) is required for other roles of H2AX in

protecting genetic integrity, e.g. regulation of G2/M checkpoint (Fernandez - Capetillo *et al.*, 2002) or promotion of sister chromatid recombination (Xie *et al.*, 2004). In favour of the second possibility, it has been shown that many aspects of DDR are attenuated by differentiation. For example, NER, BER and HR are more proficient in cycling than in terminally differentiated cells, and the repair of transcribed genes may be the minimal requirement of nonproliferating cells. (Fortini and Dogliotti, 2010; Nouspikel, 2007). It is hard to imagine that luminal epithelial cells of prostate tissue would tolerate high amounts of unrepaired DSBs, especially if they are constantly introduced by AR signaling (Haffner *et al.*, 2010). Maybe it is sufficient to either reseal (even incorrectly) broken DNA ends on euchromatin regions with ATM - independent NHEJ, or undergo apoptosis. It is difficult to speculate how the reduced levels of H2AX would affect its role in apoptosis, other than the fact that they clearly do not prevent luminal cells from undergoing this form of programmed cell death.

1.4. p53 responses after DNA damage (I, II, III)

1.4.1. DSBs and p53 response in ex vivo - cultures

p53 works as a cellular alarm clock. It constantly monitors intracellular events and responds to many forms of stress by regulating transcription of numerous genes. DSBs are known to stabilize and activate p53 via several ATM - dependent phosphorylation events that release p53 from efficient proteosomal degradation, but there is considerable variation between different tissues and cell types. In mouse tissues after IR, ATM is required for p53 – mediated apoptosis in thymus, spleen and brain (Gurley and Kemp, 2007). In small intestine, lack of AMT results only in a slight delay in p53 induction and apoptosis, and in hair follicle epithelial cells ATM is dispensable (Gurley and Kemp, 2007). We were interested in how potent p53 inducing DNA damaging agents would act in prostate and seminal vesicle tissues, and whether the guardian of the genome would be more vigilant in the tumor resistant seminal vesicles.

We quickly noticed that treatment with IR or doxorubicin resulted in only moderate p53 stabilization and p21 induction in prostate tissue, although some patients seemed to respond more strongly than others (I, Figures 2, 3 and S7). Another TOP2 - inhibitor etoposide, in spite of the distinctive induction of γH2AX, was unable to stabilize p53 in prostate. The results were similar in seminal vesicle *ex vivo* - tissues. Treatment with IR led only to moderate stabilization of p53 and p21, and more importantly, there were no significant differences between prostate and seminal vesicles (III, Figure 3B and 3D). We therefore concluded, that DSBs signaling via ATM alone is not sufficient for full p53 activation, and

that ATM - γH2AX - 53BP1 signaling and p53 do not differ significantly in differentiated cells of the prostate and seminal vesicles.

Interestingly, p53 stabilization occurred mostly in epithelial but not in stromal cells of both prostate and seminal vesicles (I, Figure 4 and III, Figure 3). This is somewhat surprising, as under cell culture environment p53 is readily stabilized in normal human fibroblasts. Moreover, after IR and also to some extent after other DNA damaging treatments, it seemed that basal cells were able to launch the most extensive p53 induction, while the highest levels of p21 were detected in secretory cells. It is difficult to explain these findings based on our current knowledge on p53, since they are a part of the unresolved context dependency - problem (Zilfou and Lowe, 2009). We do not know enough about the expression patterns of the many proteins interacting with p53, including proteins that contribute to p53 stability (E3 - ligases, DUBs and other enzymes that modify p53 post - translationally via phosphorylation, acetylation, methylation or sumoylation), DNA binding and transactivation capabilities (such as Mdm4). It is also likely that we have only started to understand the many roles of p53 - regulated genes, such as p21, the canonical guardian of the G1 cell cycle checkpoint. It is hard to connect the role of p21 induction in postmitotic secretory cells with the maintenance of G1 arrest. It could rather be required to suppress apoptosis of secretory cells after DNA damage.

1.4.2. Other forms of DNA damage and p53

The fact that DSB response does not evoke strong p53 activation in differentiated cells of prostate and seminal vesicles does not mean that p53 is redundant in these tissues. Experiments where degradation of p53 was inhibited either by releasing it from Mdm2 with Nutlin - 3 or by blocking proteosomal function with MG132, led to its accumulation (II, Figure S11 and I, Figure S8B). They indicated that p53 is constantly being transcribed and degraded and can be stabilized in prostate epithelium. Furthermore, TOP1 - inhibitor CPT invariably induced high levels of p53 and p21 in prostate and seminal vesicle tissue (I, Figures 2, 3, 4, S6 and III, Figure 3), as well as by treatments with actinomycin D (I, Figure S8A) and Dichloro - ribofuranosylbenzimidazole (DRB). Common to all these drugs is that they are potent inhibitors of transcription. We therefore suggest that transcriptional stress in differentiated cells leads to p53 stabilization.

There has been some disagreement over whether IR or DSBs affect transcription. It is possible that ribosomal RNA transcription is transiently reduced by DSBs in an ATM - dependent manner (Kruhlak *et al.*, 2007). Recent evidence suggests that ATM mediates histone H2A ubiquitylation via RNF8 and RNF168 (Doil *et al.*, 2009; Mailand *et al.*, 2007; Stewart *et al.*, 2009) to inhibit transcription in chromosomal regions close to DBS, but does not cause a global reduction in transcription (Shanbhag *et al.*, 2010). Thus, it is possible that the stronger or more universal the disturbance in transcription is, the better is

stabilization of p53. Perhaps doxorubicin, being a DNA intercalating agent, is able to inhibit transcription more potently than etoposide that is thought to affect TOP2 via protein - protein interactions.

There is some evidence on how p53 could detect transcriptional stress. One possibility is that nucleolus, the transcription site of ribosomal RNA, acts as a stress sensor for p53, and impaired nucleolar function is the common signal for stabilization of p53 (Rubbi and Milner, 2003). Other option is that p53 can sense stalling of RNA polymerase II more directly via its interactions with components of the transcriptional machinery (Laptenko and Prives, 2006; Riley *et al.*, 2008). Stabilized p53 could then restore intracellular balance by activating or repressing transcription of appropriate target genes according to a cell type and the nature of DNA damage (Zhao *et al.*, 2000).

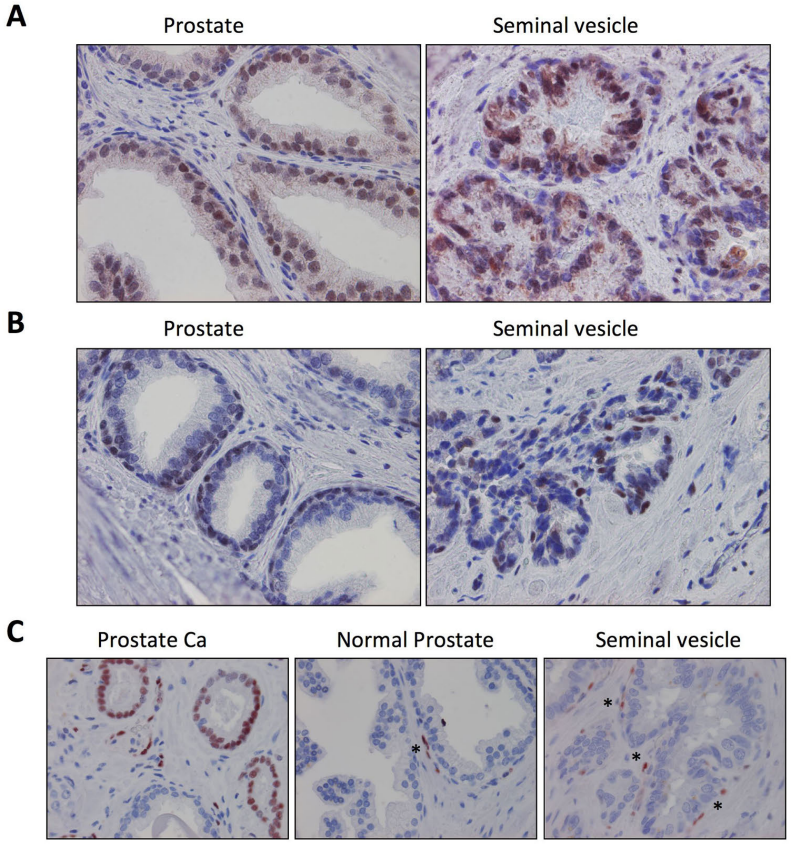
1.5. The prerequisites of *TMPRSS2 - ERG* - fusions in prostate and seminal vesicles (III and unpublished data)

According to recent publications, TMPRSS2 - ERG - fusions arise not as random events but as a result from misrepair of certain transcription - associated DSBs (Bartek *et al.*, 2010). All the specific requirements for the formation of gene fusions in prostate cancer have probably not yet been characterized, but several proteins have been implicated. The initial DSBs are formed by the concerted action of AR and TOP2B (Haffner *et al.*, 2010). These recombinogenic breaks activate ATM - pathway, and are repaired by NHEJ - and poly (ADP - ribose) polymerase 1 (PARP1) –dependent manner (Haffner *et al.*, 2010; Lin *et al.*, 2009). We were therefore interested in finding out whether there would be endogenous differences in the levels of certain key recombinogenic proteins between seminal vesicles and prostate tissue.

Seminal vesicles and prostate are androgen dependent in their tissue maintenance and secretory function, and secretory cells of both express AR (**Figure 9A** and Laczko *et al.*, 2005). Under normal growth control both tissues express TOP2B mainly in their basal cell layer (**Figure 9B**), and we have not detected major differences in the early DSB - detection measured by ATM or 53BP1 activation or γH2AX levels (III, Figures 1 and 2 and S4). Further, TOP2 - inhibitors doxorubicin and etoposide are able to induce DDR at a similar scale after DSB in both tissues (I, Figures 2 - 4 and S6, S9 - S11 and unpublished data). Why then androgen signaling that results in TMPRSS2 - ERG - fusions in prostate would not do so in seminal vesicles?

Benign seminal vesicle epithelium has many characteristics of neoplastic changes, including irregularity, prominent nucleoli and presence of atypical cells (Arber and Speights, 1991). To ascertain that seminal vesicle tissue does not feature the fusion, we carried out an IHC analysis of 24 prostate and seminal vesicles samples using an antibody that has been reported to detect ERG overexpression reliably from paraffin - embedded

samples (Park *et al.*, 2010). While 8 out of the 19 prostate tumors in the set were ERG - fusion positive, we could not detect any ERG - positive epithelial regions in seminal vesicle samples, suggesting that under similar circumstances TMPRSS2 - ERG fusions occur mainly in prostate tissue (**Figure 9C and D**). More thorough investigation on the NHEJ - pathway and other DDR proteins is required to further pinpoint what renders prostate tissue so susceptible to chromosomal rearrangements. It is also possible that androgen signaling in seminal vesicles produces less or no DSBs, or that the amount of contributing endogenous or exogenous DNA damage differs markedly between the two tissues. In human tumors, TMPRSS2 - ERG - fusions arise in PIN - lesions and promote transition into cancer (Carver *et al.*, 2009). It is also possible that seminal vesicle epithelium has better proliferative control that prevents PIN - formation, and thus seminal vesicles never end up in the situation where TMPRSS2 - ERG - fusions arise in prostate tissue.



	No	ERG+	%
Prostate cancer	19	8	42
Benign prostate	5	0	0
Seminal vesicle	24	0	0

Figure 9. *AR is expressed in the secretory (A) and TOP2B in the basal cells (B) in normal human prostate and seminal vesicle tissues. ERG - overexpression (C, D) can be detected in prostate tumors but not in the normal epithelium of prostate and seminal vesicles. * indicates ERG-positive endothelial cells.*

2. DNA damage response in human primary cells of prostate and seminal vesicles

2.1. Primary cell isolation and characterization (II, III)

HPECs and human seminal vesicle epithelial cells (HSVECs) are primary cells isolated from histologically normal regions of prostate or seminal vesicles. Tissue material for our studies was collected similarly to *ex vivo* – tissue cultures, from patients undergoing radical prostatectomy due to bladder or prostate cancer. Briefly, a part of the cored tissue cylinder was dedicated for HPEC or HSVEC culture, cut into small pieces (~ 1 - 3 mm3) and placed onto collagen IV – coated cell culture dishes. Optimized culture medium that contains epithelial cell – specific growth factors and other supplements, supports the growth of a population of epithelial but not of stromal cells. We and others have successfully used this method to isolate primary prostate epithelial cells, and they are also commercially available (Garraway *et al.*, 2003; Peehl, 2005; Uzgare *et al.*, 2004). As described in the chapter on prostate tissue structure and function, primary prostate epithelial cells are considered to be transit amplifying cells.

To our knowledge, primary epithelial cells of seminal vesicles have not been investigated elsewhere. HSVECs represent a similar cell population as HPECs (III, Figure S1). They have a characteristic appearance of epithelial cells: round morphology and tendency to adhere to each other and form cobblestone like surfaces (**Figure 10.**).

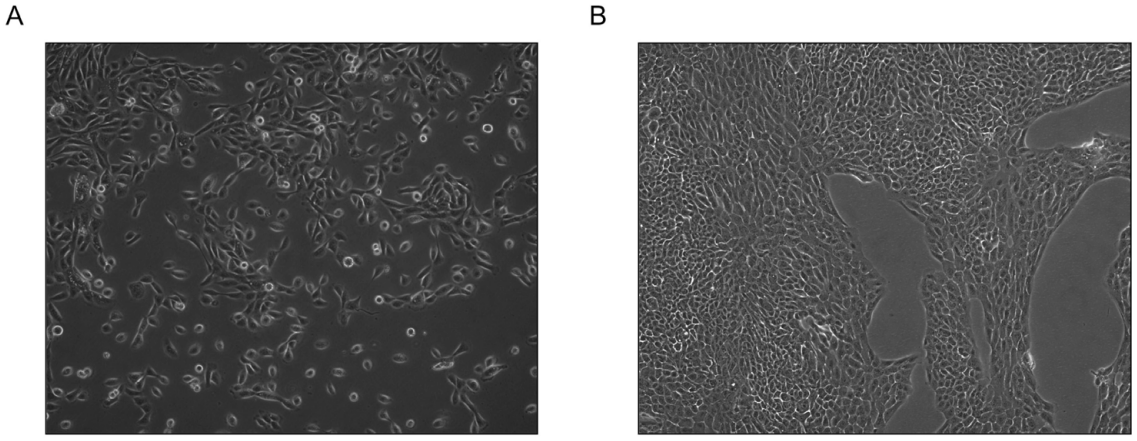


Figure 10. *(A) Early HSVECs are small, round and grow apart from each other. (B) The archetypical cobblestone appearance of more differentiated HSVECs.*

Consistent with a basal cell origin, they express basal cell markers such as epithelial cytokeratin 5 and p63 and have low levels of p27 and AR. HSVECs have a finite lifespan in

culture. They gradually lose their potential to proliferate and eventually undergo senescence. Therefore, both HSVECs and HPECs most likely represent transit amplifying cells.

2.2. Cell cycle checkpoints in HPECs and HSVECs (II, III)

HSVECs and HPECs differ dramatically in their ability to activate cell cycle checkpoints after DSBs. HPECs do not have a G1 - checkpoint arrest, they show RDS and lack also a robust G2/M arrest after IR (Girinsky *et al.*, 1995 and II, Figure 1), which is quite surprising considering that they are benign cells. This deviation in cell cycle checkpoints is not likely due to a failure in DSB detection, as early damage sensors Nbs1, Rad50 and γH2AX accumulate into DNA damage induced foci in HPECs. Equally, the effector kinase CHK2 is phosphorylated on its threonine 68, and CDC25A is degraded indicating that ATM - CHK2 - CDC25A - pathway is functional. However, the HPECs do not display inhibitory Tyr 15 phosphorylations on CDK2 and CDK1 after IR, which is consistent with their inability to arrest in cell cycle after DNA damage (II, Figure 3). HSVECs on the contrary, are able to activate both G1 and G2 –arrest. This is shown by a significant increase in the number of cells in G2 24 hours after IR, and a concomitant decrease in the number of replicating HSVECs from 17 % to less than 5 % (III, Figure 4).

Severe problems in DSB repair usually lead to increased radiosensitivity. This is especially prominent in cells with defective activation of DNA damage signaling or repair such as in ATM null cells. HPECs are not particularly radiosensitive, indicated by the lack of a significant increase in apoptotic sub - G1 - population in flow cytometry analysis after IR, so they are presumably able to repair DSBs despite their lack of cell cycle checkpoint enforcement. The checkpoints are thought to give cells more time to repair DNA damage. It is therefore possible that the lack of time forces HPECs to repair DSBs quickly at the cost of accuracy, and maybe this predisposes them to genomic translocations. As HSVECs are more proficient in their DNA - damage induced cell cycle checkpoints, it would be interesting to compare the success rate of DSB repair between HPECs and HSVECs.

2.3. p53 and the G1 arrest (II, III)

Prostate and seminal vesicle *ex vivo* – tissue slices did not differ notably in respect of p53 activation after DSBs. It was therefore surprising that there was such a difference between HSVECs and HPECs (III, Figure 5). HSVECs were able to stabilize p53 rapidly within two hours after IR, and a moderate and transient p21 induction was detectable. This pattern differs from many other primary epithelial cells, including HPECs (Girinsky *et al.*, 1995), HMECs (Huper and Marks, 2007; Meyer *et al.*, 1999), human keratinocytes (Flatt *et al.*,

1998), human bronchial epithelial cells (Gadbois and Lehnert, 1997) or mouse hepatocytes (Bellamy *et al.*, 1997). These data indicate that the regulatory network of p53 is wired differentially in HSVECs compared to many other epithelial cells. They also suggest that p53 in HSVECs may also respond more strongly to other stresses, which could offer one explanation for the question why seminal vesicle carcinomas are so exceedingly rare. On the other hand, the levels of p53 and p21 induction were not as robust as in normal human fibroblasts, although p21 expression levels responded to p53 silencing. It seems that epithelial cells cannot afford a permanent cell cycle withdrawal that has been shown to occur in human fibroblasts (Di Leonardo *et al.*, 1994).

We could also detect p53 in DNA damage induced foci that colocalized with γH2AX in both HSVECs and HPECs. Similar p53 foci have been observed in confluent G0/G1 primary fibroblasts, where they mainly consisted of serine 15 phosphorylated subpool of p53 (Al Rashid *et al.*, 2005). The role of this chromatin bound p53 has not been exhaustively clarified, but it is yet another indication of the context dependence problem of p53. Why is serine 15 phosphorylation of p53 retained in HPECs after IR, while the overall p53 stabilization is lacking? Is it only a rudiment of active ATM that is still able to phosphorylate its target protein, or does it have some irreplaceable role in primary cells? It is clearly not able to maintain functional G1 arrest in HPECs, but may be required for potentiating transactivation capabilities or apoptosis launched by p53. This view is supported by the mouse models where serine 15 phosphorylation has been constitutively prevented (Armata *et al.*, 2007; Chao *et al.*, 2003). Thymocytes of these mice are partially defective in apoptosis, and transactivation of several p53 target genes is reduced in fibroblasts without abrogation of the cell cycle arrest.

2.4. Wee1A and the G2 arrest (II, III)

Other striking feature in HSVECs that could explain their cell cycle checkpoint proficiency was their high expression levels of Wee1A, the kinase responsible for inhibitory tyrosine 15 phosphorylations on CDK1 (III, Figure 6). Wee1A expression in HSVECs was approximately 8 times higher than in HPECs and comparable to many cancer cell lines. The cause for this difference is unknown. It is possible than Wee1A protein is degraded more efficiently in HPECs. Of the 11 cell lines tested, HPECs had the lowest levels of Wee1A, and MG132 proteasome inhibitor was able to increase Wee1A levels somewhat in HPECs. Furthermore, the phosphodegron Wee1 mutant that cannot be phosphorylated by Plk1 and Cdk1 and thus escapes degradation via β - TrCP (Watanabe *et al.*, 2004; Watanabe *et al.*, 2005), was able to increase tyrosine 15 phosphorylation of CDK2 significantly (II, Figure 4). It is also possible that Wee1A expression levels in HPECs do not increase in S and G2/M phases as robustly as in HSVECs.

2.4.1. Wee1A in prostate tumors (Unpublished data)

HPECs lack DSB induced G2 arrest because of low levels of Wee1A. In normal prostate tissue, nuclear Wee1A is expressed in basal epithelial cells that unlike luminal cells have replicative potential (II, Figure 4B). Next, we asked whether Wee1A could also be a prognostic marker in prostate cancer. Preliminary studies of prostate tumors indicated that levels and localization of Wee1A were deregulated. Noting that in prostate cancer PTEN/AKT pathway is compromised with high frequency and that AKT has been shown to inactivate Wee1A by translocating it into cytoplasm (Katayama *et al.*, 2005), we carried out an extensive prostate tumor tissue microarray - analysis (in collaboration with Dr. Tapio Visakorpi, University of Tampere) and compared expression levels and localization of Wee1A, phosphorylated i.e. active AKT, and p27, which is another AKT - target altered in prostate cancer. Both primary tumors (N=258) and tumors resistant to androgen ablation therapy (N=173) were included in the study.

We were able to establish the expression patterns of Wee1 in 230 primary prostate cancer cases and in 100 androgen resistant tumors (**Figure 11.**). Most prostate tumors in both groups (71%) were negative for Wee1. Nuclear Wee1 expression was detected in 19,1 % of primary tumors and in 16 % of androgen resistant tumors, and cytoplasmic Wee1 in 9,1% and 13% of cases (unpublished observations). None of the Wee1 expression groups associated with disease progression or time to progression, so we were not able to demonstrate that Wee1 has a prognostic role in prostate cancer. Interestingly, nuclear Wee1A associated with higher Ki67 expression ($p=0.0337$) and bigger tumor size (pT2 vs. pT3, $p=0.0157$) in the group of primary tumors, which is in accordance with its elevated expression levels in S - and G2 - phases of the cell cycle. The association between nuclear Wee1A and Ki67 expression was detected also in the group of hormone refractory tumors ($p=0.0354$). Phosphorylated AKT and surprisingly, nuclear p27 correlated with high Ki67 percentage ($p<0,0001$ and $p=0,0374$), as did phosphorylated AKT and cytoplasmic Wee1A ($p=0.0365$). Phosphorylated AKT has been published to be a strong predictor of biochemical recurrence (Ayala *et al.*, 2004), but in our analysis it did not associate with poor prognosis. In conclusion, in prostate tumors nuclear Wee1A levels are upregulated in response to high proliferation rates. It therefore seems that the constraints that prevent high expression of Wee1A in HPECs are removed during tumorigenesis.

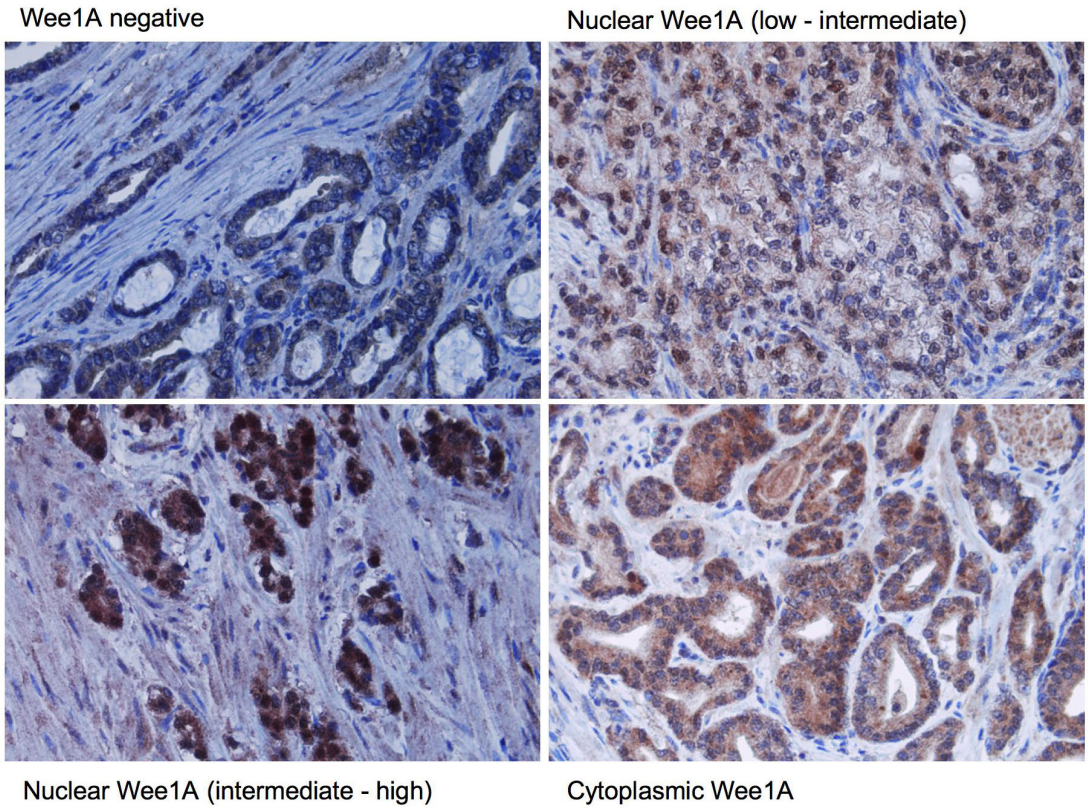


Figure 11. *Wee1A expression patterns in prostate tumors.*

Conclusions

Faulty repair of DSBs contributes to prostate tumorigenesis. Studying DSB detection and repair mechanisms in normal prostate tissue is therefore important for understanding the molecular mechanisms leading to prostate cancer. Seminal vesicles provide a good model for comparison, as they are remarkably resistant against neoplastic changes. Moreover, better understanding of the factors that prevent accumulation of genetic aberrations in seminal vesicle epithelium may reveal potential targets for cancer therapy and even cancer prevention.

In this work we studied early DDR using novel models of benign human prostate and seminal vesicles. *Ex vivo* – tissue culture offers possibilities that are not attainable by routine cell culture. They maintain the differentiated cell types, heterotopic interactions and supporting stroma, and are a good means to study the effects of therapeutic drugs on human tissue.

It is evident that many factors, including cell type and differentiation, influence DDR in ways that are sometimes surprising. We found out that terminally differentiated secretory cells express only low levels of histone H2AX, which is considered an essential component of successful DSB signaling. This suggests that either DSB signaling is less stringent in cells that no longer need to proliferate, or that H2AX has distinct, dose – dependent roles in different cell types. Function of many DDR proteins is limited into specific phases of the cell cycle, and the expression patterns of many of the newest members of the DSB signaling pathways have not been thoroughly investigated. We need a more detailed understanding of the availability of DDR factors and their biologic activity in various tissues in order to combine the different DDR pathways into a meaningful system.

Primary seminal vesicle epithelial cells proved to have more effective DSB induced cell cycle checkpoints, and p53 activation than primary prostate epithelial cells. Furthermore, the levels of Wee1A were higher in seminal vesicle epithelial cells. These findings were surprising, as we were not able to detect significant differences in the main DSB signaling pathways between the two tissues using *ex vivo* – models. These results suggest that cell proliferation in certain tissues may be a double - edged sword. It is necessary for tissue renewal after cellular damage, but at the same time may render differentiated cells more liable to acquiring genetic aberrations.

p53 activation and inhibition of Wee1A by small molecule compounds such as Nutlin - 3 and MK - 1775 are both being investigated as potential strategies for cancer therapy. Our results indicate that robust p53 activation in prostate epithelium can be achieved by administration of Nutlin - 3 and drugs causing transcriptional stress, suggesting that p53 activation is achievable in prostate tumors. On the other hand, p53 pathway activation may not be as efficient in prostate cancer as in some other cancer types due to inherent

differences in the p53 signaling pathway that already exist between benign prostate epithelium and other tissues. Similarly, the potential benefit of Wee1A inhibition in prostate cancer is uncertain, as prostate epithelial cells express only low levels of Wee1A and most prostate tumors are negative for Wee1A. The applicability of both these approaches require further studies, in which models such as primary cell and *ex vivo* – tissue culture presented here may prove to be useful.

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