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"CHK2 PHOSPHORYLATION OF SURVIVIN-ΔEX3 CONTRIBUTES TO A DNA DAMAGE-SENSING CHECKPOINT IN CANCER"

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ABSTRACT

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Mammalian cells are constantly at risk of damage to their DNA from multiple sources, including ultraviolet light, ionizing radiation (IR) and reactive oxygen species. Approximately $1X10^4$ DNA lesions are generated in a metabolically active cell each day, the majority of which are DNA single-strand breaks (SSBs), although included are the highly cytotoxic DNA double-strand breaks (DSBs). In higher eukaryotes, genomic stability is absolutely essential for healthy functioning and survival since DNA damage may induce mutations and lead to cell death. Conse quently, eukaryotic cells have evolved a number of mechanisms to monitor the integrity of their genome and repair any damaged DNA before cell cycle progression in a coordinated process that utilizes 'checkpoint' control.

CHK2 is a versatile and multifunctional kinase that regulates the cell's response to DNA damage by phosphorylating a number of distinct cellular substrates. As such, it might prevent tumour progression by averting genomic instability through DNA repair and, if this is not possible, by causing the cell to senesce or die. This correlates with human genetic studies clearly showing that CHEK2 is a multiorgan tumour susceptibility gene.

As a unique member of the Inhibitor of Apoptosis (IAP) gene family, survivin has attracted attention for its multiple roles in cell proliferation and cell death, and its abundan distribution in every human tumor, compared to normal tissues. Disparate oncogenic pathways control the differential expression of survivin in cancer, and influence its localization to multiple subcellular compartments. Accordingly, transcription of the *survivin* locus is complex, giving rise to at least five mRNA species that encode, in addition to *wild type* (WT) survivin, the variants survivin-2B, -3β, -2α and -ΔEx-3.

Structurally, survivin-2α and -3B are generated by “read-through” into intron 2, or via inclusion of an alternative exon 3B, whereas survivin-2B and -ΔEx3 originate from the insertion of an alternative exon 2B , or the skipping of exon 3, respectively.

Elucidating the function(s) of the survivin spliced variants has not been straightforward, complicated by the low levels of expression of these molecules in most cells, and the limited availability of specific reagents that discriminate between the different isoforms. For instance, survivin-2B has been reported to promote apoptosis, *in vitro*. However, low levels of survivin-2B correlate with better survival in acute myeloid leukemia, and its silencing in ovarian cancer has been linked to enhanced sensitivity to taxanes in resistant tumors. A role of the survivin isoforms in mitosis has been equally controversial, as this function has been proposed in some reports, but negated in others.

In this study, we took a multidisciplinary approach combining genome-wide bioinformatics, analysis of the DNA damage response and evaluation of primary patient samples, to investigate a potential role of a spliced survivin variant that lacks exon 3 and contains a unique –COOH terminus sequence, survivin-ΔEx3 in cancer. We show that survivin-ΔEx3 is differentially expressed in cancer, compared to normal tissues, and correlates with aggressive disease and markers of unfavorable prognosis. Unlike other survivin variants, survivin-ΔEx3 localizes exclusively to nuclei, and is phosphorylated by the DNA damage checkpoint kinase, Chk2, on residues Thr79, Ser98 and Thr127 located in its unique –COOH terminus.

ABSTRACT

Mutagenesis of Chk2 phosphorylation sites improves survivin-ΔEx3 stability in tumor cells, inhibits the expression of Ser139 phosphorylated H2AX (γH2AX) in response to double strand DNA breaks, and impairs colony formation after DNA damage, *in vitro*. In contrast, Chk2 phosphorylation on Thr68 did not affect stabilization of p53, induction of the cyclin-dependent kinase inhibitor p21, and homologous recombination-induced repair in response to DNA damage. Active Chk2 was detected at the earliest stages of the colorectal adenoma-to-carcinoma transition, persisted in advanced tumors, and correlated with increased survivin expression, *in vivo*. These data suggest that Chk2 phosphorylation of survivin-ΔEx3 contributes to a DNA damage-sensing checkpoint in tumor cells, which may affect sensitivity to anticancer therapies.

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Again and Again

to my Parents

Liliana and Gianni

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Chapter 1

INTRODUCTION

1. INHIBITOR APOPTOSIS PROTEINS (IAPS) FAMILY

The Inhibitors of Apoptosis Proteins (IAPs) family are a group of anti-apoptotic proteins that are conserved in several species [Deveraux & Reed, 1999]. These proteins are characterized by one or more 70–80 amino-acid baculoviral IAP repeat (BIR) domains. The BIR domain is the defining structural characteristic of IAP molecules. BIRs can be present in a single copy or an array of two to three repeats in the N-terminal portion of IAPs, which often include additional functional regions such as a RING or CARD (caspase-associated recruitment domain) domain near the C-terminus (Figure 1). XIAP (X-linked IAP), c-IAP1, and c-IAP2, which are the most extensively characterized of the mammalian IAPs, each contain three BIRs and a RING domain. BIRs are regions of approximately 70 amino acids that contain the variable consensus sequence C(X)2 C(X)6 (X)3 D(X)5 H(X)6 C, where X is any amino acid [Deveraux & Reed, 1999], and fold as three-stranded beta sheets surrounded by four alpha helices [Hinds *et al.*, 1999; Sun *et al.*, 1999; Sun *et al.*, 2000; Verdecia *et al.*, 2000]. The BIR helices and beta strands pack tightly to form a hydrophobic core, at the center of which lies an atom of zinc coordinated by the three cysteines and the histidine. BIRs function principally by mediating protein-protein interactions. The presence of multiple copies in a molecule can increase the affinity for a given protein as well as the range of proteins with which the IAP can interact. Interestingly, the binding properties of sequentially corresponding BIRs in multi-BIR-containing molecules is conserved, with BIR2 and -3 involved in the binding of caspases and apoptosis-regulatory molecules, and BIR1

interacting with a diverse array of signaling intermediates [Srinivasula & Ashwell, 2008]. They were first discovered in baculoviruses, a group of viruses specific to insects [Birnbaum *et al.*, 1994] and proteins containing BIR domains have been identified in a wide range of eukaryotic species and several mammalian species including mice, rats, chickens, pigs, and humans [Deveraux & Reed, 1999].

The first mammalian IAP homologue to be identified was neuronal apoptosis inhibitory protein (NAIP) (Figure 1), which was isolated during a positional cloning effort to identify the causative gene for spinal muscular atrophy [Roy *et al.*, 1995]. In contrast to the baculoviral IAPs, which possess two BIR domains and a carboxy-terminal RING zinc-finger, NAIP encodes three BIR domains and contains a very large and unique central carboxy-terminus containing a nucleotide-binding oligomerization domain (NOD) and a C-terminal leucine-rich repeat (LRR) [Koonin & Aravid, 2000] (Figure 1). The NOD and LRR domains regulate the NAIP BIR interaction with caspase-9 in a manner not seen in any other members of the family (Davoodi *et al.*, 2004). The full length NAIP binds caspase-9 only in the presence of ATP or in the absence the LRR domain, indicating that access to BIRs is spatially blocked by the C-terminal domains and that ATP-mediated conformational changes are required for caspase-9 binding.

Subsequent to the identification of NAIP, the IAP family expanded with the identification of cellular IAP1 (cIAP1, hIAP2), cIAP2 (hIAP1), and X-chromosome-linked IAP (XIAP), all of which contain three BIR domains and a carboxy-terminal RING finger [Rothe *et al.*, 1995; Duckett *et al.*, 1996; Liston *et al.*, 1996; Uren *et al.*, 1996] (Figure 1). Finally were identified survivin (with

a single BIR and the carboxy-terminal coiled-coil domain) [Ambrosini *et al.*, 1997], Livin or ML-IAP (with a single BIR and the carboxy-terminal RING finger) [Lin *et al.*, 2000; Vucic *et al.*, 2000; Kasof & Gomes, 2001], the testis-specific IAP (Ts-IAP, with a single BIR and the carboxy-terminal RING finger) [Lagace *et al.*, 2001; Richter *et al.*, 2001], and the Apollon/BRUCE (with a single BIR and the carboxy-terminal ubiquitin-conjugating enzyme) [Hauser *et al.*, 1998; Verhagen *et al.*, 2001] (Figure 1).

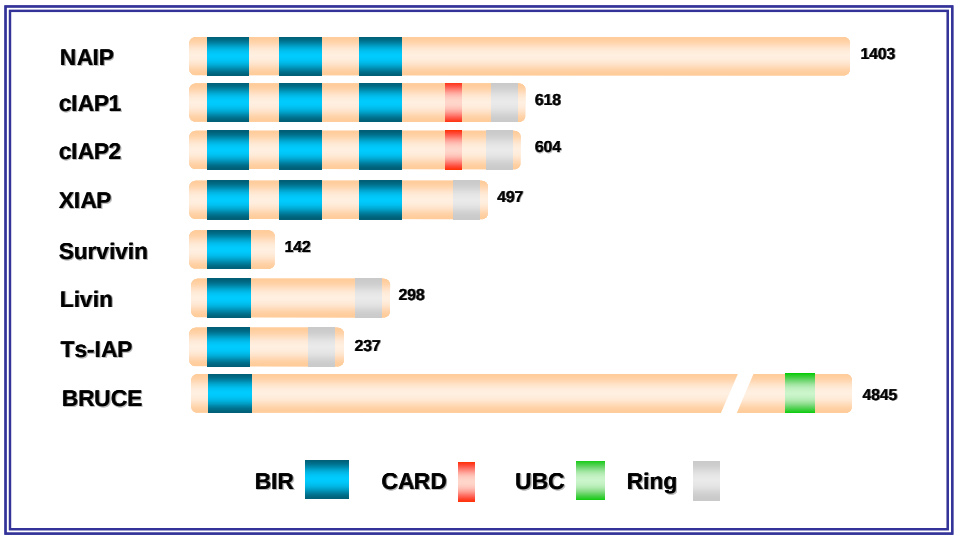

Figure 1. Schematic structure of the domains of the mammalian IAP family.
The first mammalian IAP homologue, NAIP, encodes three BIR domains and a very large and unique carboxy-terminus containing a nucleotide-binding oligomerization domain; cIAP1, cIAP2 and XIAP contain three BIR domains and a carboxy-terminal RING finger; survivin contains a single BIR and the carboxy-terminal coiled-coil domain; Livin and Ts-IAP encode a single BIR and carboxy-terminal RING finger; and BRUCE contains a single BIR and an UBC motif.

The characterization of IAP proteins suggests that they function as suppressor of cell death in a variety of tissue culture systems, including triggers of both the endogenous and exogenous pathways of apoptosis [LaCasse *et al*, 1998; Deveraux & Reed, 1999; Wright & Duckett, 2005]. It

was subsequently shown that the IAPs could directly inhibit the activity of several recombinant caspases *in vitro* [Deveraux *et al*, 1997; Roy *et al*, 1997; Maier *et al*, 2002], and structure-function studies of IAP family proteins have demonstrated a requirement for at least one BIR domain for suppression of apoptosis, although other domains found within some IAPs may also be required under certain circumstances. Several of the mammalian, fly, and viral IAPs have a RING domain located near their carboxyl termini (Figure 1). The necessity for the RING domain for suppression of apoptosis appears to depend on cellular context. In fact, some reports have indicated that the baculoviral IAPs require both amino-terminal BIR domains and the carboxy-terminal RING domain for their anti-apoptotic function in insect cells [Clem & Miller, 1994; Harvey *et al*, 1997]. By contrast, the human proteins cIAP1, cIAP2, and XIAP have been reported to retain anti-apoptotic function in the absence of their carboxy-terminal RING domains [Deveraux *et al*, 1997; Roy *et al*, 1997; Takahashi *et al*, 1998].

The BIR domains of the IAPs are the most fully characterized functional units of the IAPs. Each BIR domain folds into a functionally independent structure that chelates a zinc ion and consists of a globular head and an unstructured tail derived from the amino-terminal 'linker' region located upstream of the individual BIR domains. Specific interactions with initiator (caspase-9) and effector (caspase-3 and -7) caspases have been mapped to individual BIR domains. As a general rule, the IAPs containing multiple BIRs employ the third BIR domain to inhibit caspase-9, and the second BIR domain functions to inhibit caspase-3 and -7 [Roy *et al*, 1997; Takahashi *et al*, 1998; Maier *et al*, 2002]. As regards single BIR-containing proteins, Ts-

IAP inhibits caspase-9 [Richter *et al*, 2001], while the survivin and livin inhibit caspase-3, -7 and -9 [Lin *et al*, 2000; Vucic *et al*, 2000; Kasof & Gomes, 2001; Shin *et al*, 2001].

Among the mammalian IAPs that are known to be involved in apoptosis, X-linked IAP (XIAP) has been characterized extensively (Figure 2).

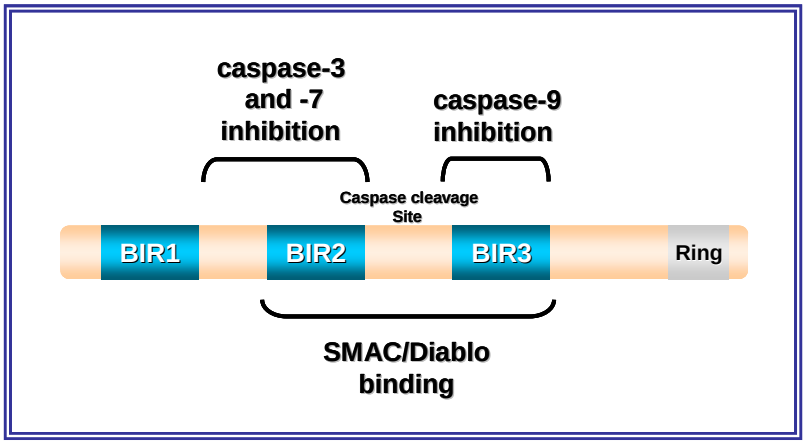

Figure 2. Map of XIAP activities and interactions.
XIAP contains three BIR domains and a C-terminal RING motif. BIR2 binds to and inhibits active caspase-3 and caspase-7, whereas BIR3 potently targets active caspase-9. No specific function has been identified for the BIR1 domain. SMAC/Diablo peptide, released from mitochondria during apoptosis, binds to a highly conserved surface groove on the BIR3 domain of XIAP, and re-activates the processed initiator and effector caspases.

This protein contains three BIR domains and a C-terminal RING motif. The second BIR domain (BIR2) specifically binds to and inhibits active caspase-3 and caspase-7, whereas BIR3 potently targets active caspase-9 [Shi, 2002]. No specific function has been identified for the BIR1 domain of XIAP. The linker segment that immediately precedes the BIR2 domain of XIAP binds to the active site of effector caspase-3 and caspase-7, hence, preventing substrate binding and subsequent catalysis. By contrast, the BIR3 domain of

XIAP employs a different method to inhibit the initiator caspase, caspase-9. Caspase-9 becomes catalytically active through a conformational change when bound by Apaf-1, and thus appears to be unique among the caspases in that there is no absolute requirement for proteolytic activation. In addition to its ability to proteolytically process caspase-3, caspase-9 can undergo a self-cleavage event in the linker region between the p20 and p10 subunits. The XIAP BIR3 domain directly binds caspase-9 via this newly exposed amino terminus and the interaction is stabilized through additional contacts with the enzyme. Using purified caspase-9 and XIAP BIR3, in the absence of Apaf-1 and cytochrome *c*, it was demonstrated that this interaction prevents caspase-9 homodimerization and stabilizes the enzyme in an inactive state similar to its monomeric form [Shiozaki *et al*, 2003]. It has been proposed that a similar change in caspase-9 morphology occurs within the apoptosome complex as a consequence of XIAP binding. Caspase-9 can also undergo further cleavage catalysed by caspase-3. This proteolytic event further increases the enzymatic activity of caspase-9, and was proposed to remove the peptide sequence that binds XIAP [Srinivasula *et al*, 2001].

In the intrinsic cell death pathway, the key event leading to the activation of caspases is the release of several pro-apoptotic proteins from the intermembrane space of mitochondria into the cytosol [Wang, 2001]. One such protein is SMAC/Diablo [Du *et al*, 2000; Verhagen *et al*, 2000]. The newly synthesized SMAC/Diablo protein contains 239 amino acids. Its N-terminal 55 residues encode the mitochondrial-targeting sequence and are proteolytically removed in the mature SMAC/Diablo protein [Du *et al*, 2000]. This cleavage results in the exposure of four hydrophobic amino acids, Ala-

Val-Pro-Ile, at the N-terminus of mature SMAC/Diablo. This tetrapeptide represents the founding member of a family of IAP-binding motifs in mammals and fruit flies [Shi, 2002]. Structural analysis reveals that this tetrapeptide motif binds to a highly conserved surface groove on the BIR3 domain of XIAP [Liu *et al*, 2000; Wu *et al*, 2000] (Figure 2). The interactions between SMAC/Diablo and IAPs requires that the N-terminus of the tetrapeptide is exposed, which explains why only the mature form of SMAC/Diablo is functional in cells. Before apoptosis, accidental activation of either caspase-9 or caspase-3 and caspase-7 might not lead to cell death because of the inhibitory effect of IAPs. During apoptosis, SMAC/Diablo is released from mitochondria and re-activates the processed initiator and effector caspases by relieving IAP-mediated inhibition.

2. SURVIVIN

Survivin is a bifunctional protein that facilitates tumour cell evasion from apoptosis and promotes mitotic progression [Altieri & Marchisio, 1999].

Survivin was discovered in 1997 by hybridisation screening of a human genomic library with the cDNA of the effector cell protease receptor-1 (EPR-1) [Ambrosini *et al*, 1997]. Survivin has a particular relationship to EPR-1: in fact its sequence is complementary to and in the reverse orientation of EPR-1.

2.1. Protein Structure of Survivin & Isoforms

The *survivin* human gene spans 14.7 kb, and is located on the telomeric position of chromosome 17 at band q25 [Ambrosini *et al*, 1998]. It comprises three introns and four exons, a TATA-less proximal promoter, and approximately 200 nt GC-rich regions upstream of exon 1 [Ambrosini *et al*, 1997]. The gene encodes a 16.5 kDa protein of 142 amino acids. The amino-terminal portion of survivin consists of three α helices (residues 14-21, 31-41, 68-80) and 3 β-sheets (residues 43-45, 55-58, 61-64), which closely resemble the BIR domain that is conserved in the IAP family [Ambrosini *et al*, 1997; Chantalat *et al*, 2000] (Figure 3). Differently from other IAPs, survivin contains only one BIR and a carboxy-terminus coiled-coil, but no RING finger or other identifiable domain [LaCasse *et al*, 1998].

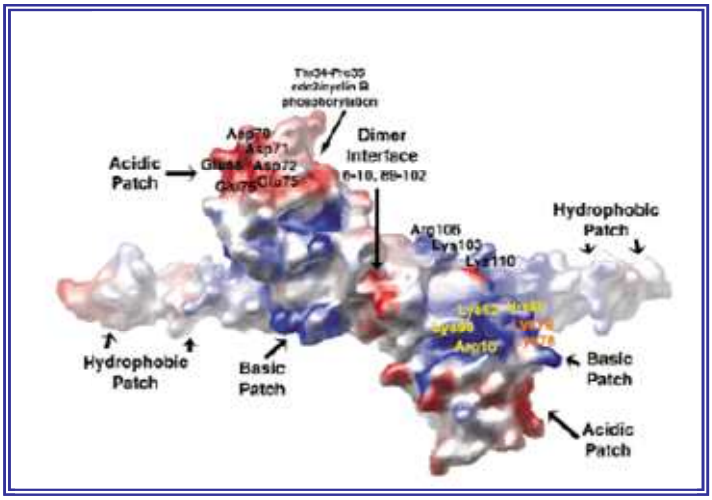

Figure 3. Representation of survivin protein structure.
The amino-terminal portion of survivin consists of three α helices (residues 14-21, 31-41, 68-80) and 3 β-sheets (residues 43-45, 55-58, 61-64), which closely resemble the BIR domain. Differently from other IAPs, survivin contains only one BIR and a carboxy-terminus coiled-coil, but no RING finger or other identifiable domain. [Adapted from Verdecia *et al*, 2000]

Crystal structure analysis of survivin revealed that it exists as a dimer, with the two BIR domains forming a "bow-tie-shape" [Chantalat *et al*, 2000] (Figure 4). The carboxy-terminal helix is not involved in the dimerization of survivin but extends outward from the entwined BIR structure [Chantalat *et al*, 2000].

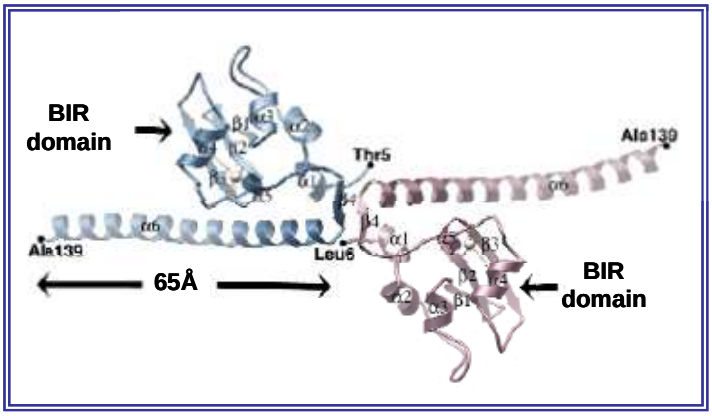

Figure 4. Representation of the survivin dimer.
[Adapted from Verdecia *et al*, 2000]

The survivin gene is subjected to alternative splicing (Figure 5). Human survivin gene has four dominant (1, 2, 3, and 4) and two hidden (2B and 3B) exons. Alternative splicing of its pre-mRNA give rises to four different mRNAs, which encode four distinct proteins, survivin, survivin-2B, survivin-ΔEx3 [Mahotka *et al*, 1999], and survivin-3B [Badran *et al*, 2004] (Figure 5).

Figure 5. Schematic representation of the alternative splicing variants of survivin.

Human survivin gene has four dominant (1, 2, 3, and 4) and two hidden (2B and 3B) exons. Alternative splicing of its pre-mRNA produces different mRNAs, which encode five distinct proteins, survivin, survivin-2B, survivin-ΔEx3, survivin-3B, and survivin 2α.

Survivin (142 aa) is derived from exons 1-4; in survivin-2B (165 aa), the BIR motif is interrupted by the in-frame insertion of a novel sequence of 23 aa derived from a part of intron 2 that is retained as cryptic exon (Figure 5);

survivin-ΔEx3 (137 aa) is derived from the joining of exons 2 and 4, skipping exon 3 (Figure 5).

Deletion of exon 3 in survivin-ΔEx3 results in a frame shift after residue ^{73}P with truncation of the BIR domain in addition to a new COOH-terminal protein segment, which in turn might affect functional properties such as subcellular localization. Infact, the carboxy-terminal end of survivin-ΔEx3 contains a bipartite nuclear localization signal, not present in survivin, which mediates its strong nuclear accumulation [Rodriguez *et al*, 2002]. Another splice variant, survivin-3B comprises 5 exons including novel exon 3B derived from a 165-bp long portion of intron 3. Acquisition of a new in-frame TGA stop codon within the novel exon 3B results in an open reading frame (ORF) of 363 nucleotides, predicting a truncated 120 amino acid protein. Expression of survivin-3B was detected in human colon and gastric adenocarcinoma cell lines as well as mononuclear cells prepared from patients with MDS and AML. It contains a single BIR, which is critical for apoptosis inhibition. However, survivin-3B lacks a carboxyl-terminal coiled-coil domain, suggesting that survivin-3B may not be associated with G2/M phase. These data indicate that the function of survivin-3B may be different from that of regular survivin. [Badran *et al.* 2004]. Morever, an additional splice variant, survivin 2α, has been identified [Caldas *et al*, 2005]. Structurally, the transcript consists of 2 exons: exon 1 and exon 2, as well as a 3' 197 bp region of intron 2 (Figure 5). Acquisition of a new in-frame stop codon within intron 2 results in an open reading frame of 225 nucleotides, predicting a truncated 74 amino acid protein. Survivin 2α is expressed at high levels in several malignant cell lines and primary tumors. Functional

assays show that survivin 2α attenuates the anti-apoptotic activity of survivin. [Caldas *et al*, 2005a].

Little is known about the differential functions of survivin alternative splice forms, which are generally expressed at lower levels than the wild-type survivin. However, it has been reported that the expression of survivin-2B is predominant in some neuroblastoma with a good prognosis, but it is expressed at low levels in most malignant tissues [Islam *et al*, 2000]. In renal cell carcinomas (RCC), while the expression levels of survivin and survivin-ΔEx3 did not show a decrease in late tumour stages in comparison with the early and intermediate tumour stages in 57 clinical RCC samples, survivin-2B expression was significantly decreased in late tumour stages [Mahotka *et al*, 2002a], indicating a possible unfavourable role of these splicing variant in the development of this tumour type. In gastric cancer, the expression of survivin-ΔEx3, survivin-2B, and survivin (dominant transcript) was found in clinical samples, irrespective of their histological type, grade, or stage [Krieg *et al*, 2002; Meng *et al*, 2004]. However, survivin-2B expression was significantly decreased in later tumour stage in comparison with early stage [Krieg *et al*, 2002; Meng *et al*, 2004], and inversely correlated with tumour differentiation and invasion [Meng *et al*, 2004]. The expression of survivin and survivin-ΔEx3 remained unchanged in different stages of cancer [Krieg *et al*, 2002], and the expression level of survivin-ΔEx3 is inversely correlated with apoptotic index [Meng *et al*, 2004]. It was also reported that survivin-2B expression was dominant in benign brain tumours in comparison with the malignant ones [Yamada *et al*, 2003], and that survivin-ΔEx3 expression was prominent in comparison with survivin-2B expression in survivin-expressing

acute lymphocytic leukaemia and chronic lymphocytic leukaemia patient bone marrow samples [Nakagawa *et al*, 2004].

Preliminary data would suggest that heterodimerisation of survivin with survivin-ΔEx3 is essential for the inhibition of mitochondrial-dependent apoptosis [Caldas *et al*, 2005b], further suggesting a possible anti-apoptotic role for survivin-ΔEx3. In addition, the different subcellular localisation of survivin-ΔEx3 (in nucleus) and survivin-2B (in cytoplasm) [Mahotka *et al*, 2002b] suggests their potential different functions. These observations indicate that survivin-ΔEx3 and survivin-2B may play an opposing role in tumour progression and tumorigenesis. Moreover, it has been demonstrated in exogenous expression assays that survivin 2α attenuates the anti-apoptotic activity of survivin [Caldas *et al*, 2005a]. More recently, it was reported that despite their ability to interact with wild-type survivin, alternative splice isoforms such as survivin-ΔEx3 and survivin-2B do not play a role in mitosis since they do not localize with the chromosomal passenger complex *in vivo* as a consequence of their reduced affinity for the survivin partner Borealin [Noton *et al*, 2006]. Moreover, these splice variants cannot rescue cell proliferation inhibited by RNAi-mediated survivin depletion [Noton *et al*, 2006].

Bladder cancer development and progression are complicated processes. Differen studies have shown the importance of the survivin gene in the development and progression of bladder tumors [Hou *et al,* 2006, Wang *et al,* 2008]. After siRNA interference inhibited the expressions of survivin, survivin-ΔEx3, and survivin-2B in bladder cancer, downregulating these 3 genes simultaneously, rather than downregulating one, enhanced cell apoptosis and

increased sensitivity of tumor cells to chemotherapeutic drugs [Wuttig *et al,* 2007].

He *et al.* successfully detected the mRNA expressions of survivin, survivin-ΔEx3, and survivin-2B in 60 bladder transitional cell carcinoma (BTCC) tissue samples and their mRNA expression levels in the experimental group were all significantly higher than those in the control group. It appears that these two splice variants of survivin played certain roles in BTCC development and progression. Further analysis showed that the expression levels of survivin mRNA were positively correlated to pathologic grade and depth of tumor invasion, suggesting that survivin influenced the progression of bladder tumor. In addition, there was a positive correlation trend between the expression level of survivin-ΔEx3 and pathologic grade. The expression level of survivin-ΔEx3 also increased significantly with a rise in pathologic grade , suggesting that survivin-ΔEx3 is similar to survivin in affecting bladder tumor progression. Moreover, the expression level of survivin-ΔEx3 significantly increased in grade III BTCC, indicating that survivin-ΔEx3 not only had antiapoptosis activity but also promoted the proliferation of tumor vascular endothelial cells [Caldas *et al,* 2007], which accelerated bladder cancer progression. This finding suggested that survivin-ΔEx3 can be used as a potential index to evaluate bladder cancer prognosis and also a selectively blocked target in the future.

In a recent study, the expression level of survivin and its splice variant, survivin-ΔEx3, in tumoral, non-tumoral and adjacent non-neoplastic tissues was analyzed. In previous reports on the expression of the survivin in thyroid tumors, it was revealed that the high expression of survivin was correlated

with thyroid tumors [Ito *et al,* 2003; Xiang *et al,* 2007], but a comprehensive data about the expressional level of survivin and its splice variants in tumoral and non tumoral thyroid nodules has been very rare until now.

It has been reported that the survivin and its splice variants are highly expressed in several human carcinoma including lung, breast, colon and oesophageal compared with normal tissues [Futakuchi *et al,* 2007; Zaffaroni *et al,* 2005; Yamada *et al,* 2003; Atlasi *et al,* 2009; He *et al,* 2009; Ryan *et al,* 2005; Suga *et al,* 2005]. Moreover Ito *et al.* [2003], reported the high expression level of survivin in human thyroid tumors using immunohistochemistry technique.

Vandghanooni *et al.* [2011] showed a significantly high expression level of survivin in malignant papillary thyroid carcinoma compared with others. They also found a significant increased expression level of survivin-ΔEx3 in malignant tumors compared with adjacent non-neoplastic tissues and non-tumoral ones. In addition, survivin-ΔEx3 was significantly elevated in malignant tumor tissues compared with benign tumors.

Survivin exhibits cell cycle-dependent expression at mitosis. This requires canonical CDE/CHR boxes in the proximal survivin promoter [Kobayashi *et al*, 1999; Badie *et al*, 2000] acting as G_1-repressor elements to shut down gene transcription in interphase cells [Kobayashi *et al*, 1999]. Survivin levels are regulated by rapid changes in protein stability. Poly-ubiquitination on multiple Lys residues and proteasomal-dependent destruction has been proposed as a mechanism to maintain low levels of survivin in interphase cells [Zhao *et al*, 2000], thus further enhancing cell cycle periodicity. In addition, mitotic phosphorylation of survivin on Thr34 by $p34^{cdc2}$-cyclin B1 has been

associated with increased survivin stability at metaphase [O'Connor *et al*, 2002]. It has been suggested that this pathway is dominant in normal cells and constitutes the primary function of survivin in adult tissues [Yang *et al*, 2004; Altieri, 2006].

Other non-cell-cycle-dependent mechanisms driving *survivin* gene transcription independent of mitosis have been described, which involve tissue patterning circuits (Wnt/β-catenin) [Kim *et al*, 2003], cytokine activation (STAT3) [Gritsko *et al*, 2006], costimulatory messages (OX-40) [Song *et al*, 2005] and pleiotropic signaling mechanisms (AkT, NFkB) [Mitsiades *et al*, 2002] that are operative during development and generally up-regulated in cancer cells [Vogelstein & Kinzler, 2004] and can explain survivin overexpression in the large majority of human tumours. It has been recently suggested that these non-cell-cycle-dependent pathways are dominant in tumours. This hypothesis also relies on the fact that a transgenic mouse model expressing the green fluorescent protein reporter gene under the control of the minimal survivin promoter demonstrated that expression of survivin in development and tumour formation is largely independent of cell-cycle-dependent transcription of the survivin gene at mitosis [Xia & Altieri, 2006]. The fraction of survivin produced through these non-cell-cycle–dependent mechanisms mediates apoptosis inhibition through intermolecular cooperation with cofactors including the hepatitis B virus X interacting protein [Marusawa *et al*, 2003], a target of the oncogenic viral HBX protein, and XIAP [Dohi *et al*, 2004a].

2.2. Survivin & Inhibition of Apoptosis

Survivin plays an important role in the suppression of apoptosis by either directly or indirectly inhibiting the activity of caspases (Figure 6).

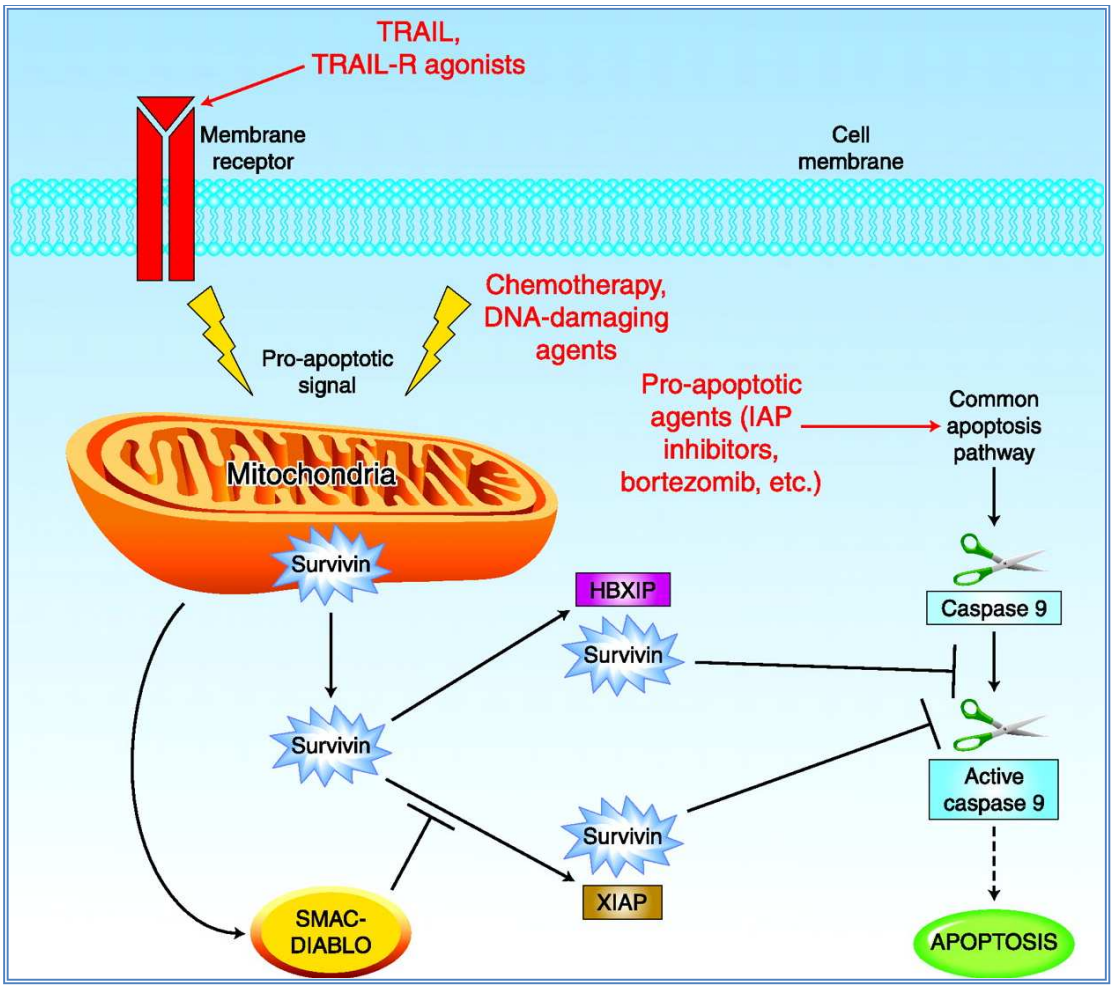

Figure 6. Function of survivin as inhibitor of apoptosis.

Upon activation of proapoptotic cell signaling, survivin is released from the mitochondria in the cytosol and inhibits active caspase-9. This function requires association with the hepatitis B X-interacting protein and/or with X-linked IAP and is inhibited by Smac/Diablo (Adapted from Mita *et al, Clin Cancer Res, 2008*).

However, the role of survivin in apoptosis inhibition has been the subject of controversy. Initially, survivin and other IAPs were postulated to selectively bind to and promote the degradation of active caspase-3, caspase-7, and caspase-9 [Altieri, 2003]. In support of this model, it was shown that survivin is inhibited by Smac/DIABLO, thus placing survivin in a central position in the dynamic balance of proapoptotic and antiapoptotic factors [Dohi *et al*, 2004a]. However, this model was challenged by the observations that survivin lacks the structural motifs that mediate binding to caspases that are present in other IAPs and that the role of survivin-like orthologues in *Caenorhabditis elegans* and other organisms seemed exclusively involved in cell division but not in cytoprotection [LaCasse *et al,* 1998]. Later experiments indicated that survivin inhibits active caspase-9 but not active caspase-3 and caspase-7 and that the inhibition of caspase-9 requires a cofactor, the hepatitis B X-interacting protein [Marusawa *et al,* 2003]. Additionally, it was suggested that the antiapoptotic role of survivin could be mediated by its association with X-linked IAP via their conserved baculovirus–inhibitor of apoptosis domain, thus increasing X-linked IAP stability and leading to the synergistic inhibition of caspase-9 activation [Dohi *et al*, 2004b]. Therefore, the ability of survivin to inhibit apoptosis seems likely although its mechanism of action may be more sophisticated than direct caspase inhibition and could involve cooperation with other molecules (such as hepatitis B X-interacting protein and X-linked IAP).

2.3. Survivin & Cell Division

The role of survivin in cell division is unanimously accepted. During the cell cycle, survivin localizes to various components of the mitotic apparatus [Vagnarelli & Earnshaw, 2004] (Figure 7).

The tightly cell-cycle–dependent control of the synthesis and degradation of survivin in normal tissues strongly supports its role in mitotic regulation. During mitosis, survivin functions in a narrow time window at metaphase and anaphase and localizes to two main subcellular pools. One pool of survivin is directly associated with polymerized tubulin. This pool involves centrosomes, microtubules of the metaphase and anaphase spindle, and the remnants of the mitotic apparatus, and suggests a regulation of microtubule dynamics [Li *et al*, 1998; O'Connor *et al*, 2002; Giodini *et al*, 2002; Altieri, 2006]. A second pool of survivin localizes to the kinetochores of metaphase chromosomes. In this pool, survivin is associated with regulators of cytokinesis, such as Aurora B kinase, INCENP, and Borealin/Dasra [Bolton *et al*, 2002; Wheatley *et al*, 2001; Sampath *et al*, 2004; Gassmann *et al*, 2004], which supports a role for survivin as a subunit of the chromosomal passenger complex that is essential for proper chromosome segregation and cytokinesis [Lens *et al*, 2006]. Although the functions of survivin as a regulator of microtubule dynamics and chromosomal passenger protein may seem incompatible, a recently proposed theory of survivin as a central regulator of spindle formation may reconcile the two. According to this model, survivin mediates the proper targeting of chromosomal passenger proteins to

kinetochores and, in addition, stabilizes the microtubules, thus contributing to bipolar spindle formation [Altieri, 2006].

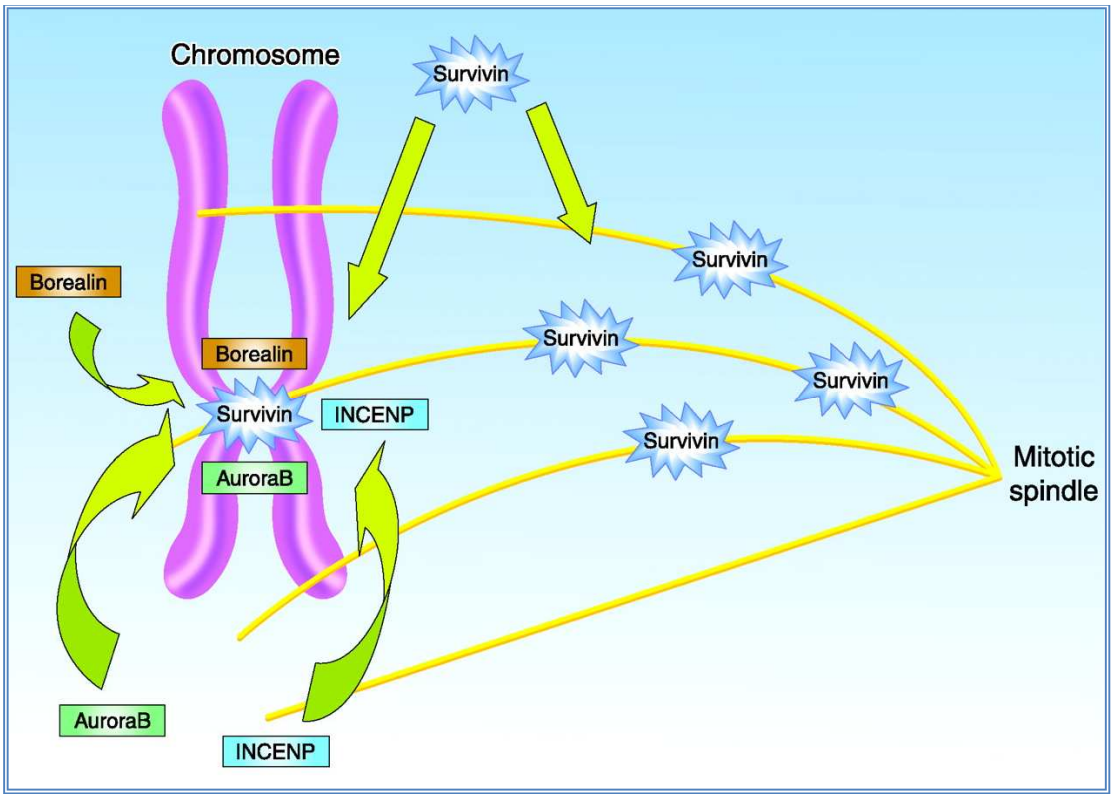

Figure 7. Function of survivin in mitosis.

Survivin is a component of the chromosomal passenger complex that is essential for proper chromosome segregation and cytokinesis. Additionally, a distinct pool of survivin is directly associated with polymerized tubulin and contributes to the regulation of microtubule dynamics. (Adapted from Mita *et al, Clin Cancer Res, 2008*).

2.4. Regulation of Survivin Function

A critical requisite for survivin function was identified in the phosphorylation on threonine 34 [O'Connor *et al*, 2000]. The only kinase recognized to phosphorylate survivin is $p34^{cdc2}$-cyclinB1, a cyclin-dependent kinase that is only active during certain points in the cell cycle [O'Connor *et al*, 2000]. The $p34^{cdc2}$-cyclin B1 was shown, *in vivo*, to physically associate with survivin and to phosphorylate survivin on Thr^{34} during mitosis. A direct link between survivin and $p34^{cdc2}$-cyclin B1 was independently confirmed using array technology in gene-profiling studies of large-cell non-Hodgkin's lymphoma; it was associated with an activated B-cell phenotype and unfavourable disease progression [Kuttler *et al*, 2002]. Mutants of $p34^{cdc2}$-cyclin B1 prevent survivin phosphorylation, and the enzyme can be co-immunoprecipitated with survivin in cells that have been synchronized in mitosis. Although other members of the cdc2 family of kinases may also associate with survivin [Suzuki *et al*, 2000], they are active only during certain cell cycle transitions, and thus the activity of survivin as an apoptosis-blocking protein is likely restricted to dividing cells. The p53-dependent repression of cdc2 expression has been demonstrated during G_2-arrest [Passalris *et al*, 1999], suggesting the hypothesis that p53 loss is associated with greater phosphorylation of survivin and thus more complete suppression of apoptosis.

Expression of non-phosphorylatable survivin $Thr^{34}\rightarrow$Ala prevented phosphorylation of endogenous survivin, resulting in apoptosis of various cancer cell types [O'Connor *et al*, 2000], and suppressed cell growth *in vivo*

[Grossman *et al*, 2001; Mesri *et al*, 2001]. Moreover, inhibitors of cyclin-dependent kinases (cdk) such as flavopiridol or the more $p34^{cdc2}$-specific inhibitor, purvalanol A, were tested in tumour cells arrested at mitosis with paclitaxel, which induces hyperphosphorylation of survivin on Thr^{34} [Zaffaroni *et al*, 2002]. Sequential administration of cdk inhibitors resulted in escape from the mitotic block imposed by paclitaxel, marked activation of mitochondrial-dependent apoptosis and anticancer activity *in vivo* [O'Connor *et al*, 2002].

A physical interaction with the molecular chaperone heat shock protein 90 (Hsp90), which involves the Hsp90 ATPase domain and the survivin BIR domain, was shown to be essential for the stability and function of survivin. In fact, targeted antibody-mediated disruption of the survivin-Hsp90 complex in cancer cells resulted in proteasomal degradation of survivin, mitochondrial-dependent apoptosis, and mitotic arrest [Fortugno *et al*, 2003].

2.5. Survivin Expression in Normal & Tumour Tissues

One of the most significant features of survivin is its differential expression in cancer versus normal tissues. Survivin expression in normal tissues is developmentally regulated and the protein was found to be absent or low in most terminally differentiated tissues [Ambrosini *et al*, 1997]. However, recent studies tend to attribute a role to survivin in regulating the function of normal adult cells [Fukuda *et al*, 2006] including vascular endothelial cells [Mesri *et*

al, 2001], polymorphonuclear cells [Altznauer *et al*, 2004], T cells [Xing *et al*, 2004], erythroid cells [Gurbuxani *et al*, 2005], and haematopoietic progenitor cells [Fukuda & Pelus, 2001]. Moreover, survivin expression was reported in adult liver cells [Deguki *et al*, 2002], gastrointestinal tract mucosa [Chiou *et al*, 2003] and ovarian granulosa cells [Wang *et al*, 2004]. However, although survivin is expressed in normal tissues characterized by self-renewal and proliferation, its expression is significantly lower than in transformed cells. In fact, several studies have demonstrated strong survivin expression in most human solid tumour types and haematologic malignancies [Altieri, 2003]. Expression of survivin has also been detected in a variety of benign and preneoplastic lesions including polyps of the colon, breast adenomas, Bowen's disease and hypertrophic actinic keratosis [Altieri, 2003], suggesting that re-expression of survivin may occur early during malignant transformation or following a disturbance in the balance between cell proliferation and cell death. The up-regulation of survivin at the transcriptional level in human tumours has been confirmed in genomewide searches, which indicated survivin as the fourth top 'transcriptome' in cancers of various histology [Velculescu *et al*, 1999]. Moreover, global deregulation of the *survivin* gene mediated by oncogenes such as STAT3 [Gritsko *et al*, 2006], E2F [Jiang *et al*, 2004], and activated H-Ras [Sommer *et al*, 2003; Fukuda & Pelus, 2004], or by loss of tumour suppressors like wild type p53 [Hoffman *et al*, 2002] or the adenomatous polyposis coli protein [Zhang *et al*, 2001], seems to be responsible for the enhanced expression of survivin in tumours.

Growing evidence suggests that survivin expression in cancer cells is associated with clinicopathologic variables of aggressive disease and may

represent an important prognostic marker for patient outcome. In fact, several studies on different types of solid tumours and haematologic malignancies showed that high levels of the protein were predictive of tumour progression in terms of either disease-free or overall survival [Altieri, 2003]. In several neoplasms the association with tumour progression was corroborated in the context of a comprehensive analysis of gene-expression profiling by DNA microarray or PCR-based assay. As it is possible to immunohistochemically distinguish two intracellular pools of survivin, a nuclear and a cytosolic one, the prognostic significance of the protein has been analyzed in some studies as a function of its intracellular localization, and inconsistent and sometimes contrasting results have been obtained regarding the prognostic value of nuclear survivin expression [Fengzhi *et al*, 2005]. The different prognostic value of survivin may reflect differences in methods to detect its expression and/or a differential expression of survivin splice variants.

2.6. Survivin as a Determinant of Treatment Resistance

Considering that apoptosis is the primary mode of cell death induced by several classes of chemical and physical agents commonly used in cancer therapy, it has been hypothesized that survivin expression/up-regulation could be crucial in determining the chemo- and radio-sensitivity profiles of tumour cells. This possibility is supported by several experimental evidences

demonstrating that survivin is able to counteract a broad range of different apoptotic stimuli.

Giodini *et al* [2002] first reported that infection of HeLa cells with an adenoviral vector expressing survivin suppressed apoptosis induced by taxol. Successively, studies performed on human ovarian carcinoma cell lines and clinical specimens clearly indicated that survivin is involved in regulating cell sensitivity to taxanes. Specifically, the OAW42 and IGROV-1 human ovarian cancer cell lines were transfected with the human survivin cDNA. Stable transfection with survivin cDNA was able to protect these cells from the cytotoxic effects induced by paclitaxel and taxotere, with IC_{50} values for the survivin-transfectant cell populations 4-6-fold those of the control cells [Zaffaroni *et al*, 2002]. Zhang *et al* [2005] showed that forced expression of wild type survivin in human prostate cancer cell lines increased the resistance to taxol *in vitro* and *in vivo*. In addition, in the clinical setting, in advanced ovarian cancer patients receiving a paclitaxel+platinum-based regimen, the overexpression of the anti-apoptotic protein correlated with a lower clinical or pathologic complete remission rate (with respect of those treated patients where survivin expression was found to be low or absent) [Zaffaroni *et al*, 2002].

It has been reported that taxol-induced microtubule stabilization and mitotic arrest increase the expression of survivin, which engenders a cell survival pathway to counteract taxol-induced apoptosis [O'Connor *et al*, 2002]. Interestingly, using a taxol-resistant ovarian cancer cell clone, PTX10, with a β-tubulin mutation at the taxol binding site, Zhou *et al* [2004] found that taxol treatment failed to induce mitotic arrest and survivin expression.

However, the finding that taxol induced an apoptotic response in these cells suggests that mitotic arrest is not strictly required for taxol-induced apoptosis. It is also possible that the mitotic survival pathway is not the only one by which cancer cells counteract taxol-induced apoptosis. In fact, Ling *et al* (2004) reported that induction of survivin by taxol in MCF-7 cells is an early event and is independent of taxol-mediated G_2/M arrest, suggesting a role for survivin in taxol resistance not only during mitosis but outside of the mitotic checkpoint as well. There is evidence that the mTOR pathway, which constitutes a sensor network for stress conditions, is involved in resistance to paclitaxel by increasing survivin levels [Vaira *et al*, 2006]. In fact, it has been recently reported that IGF-1-mediated mTOR activation in prostate cancer cells positively modulated survivin levels by favoring stabilization and translation of a survivin mRNA pool, and that mTOR inhibition with rapamycin, alone or in combination with paclitaxel, abolished survivin increase. Consistent with a critical reduction of the anti- apoptotic threshold maintained by survivin, the paclitaxel plus rapamycin combination was more effective than either treatment in reducing cell viability of in the presence of IGF-1 [Vaira *et al*, 2006].

An increase in survivin expression has also been reported in prostate cancer [Nomura *et al*, 2005] and thyroid cancer [Tirro *et al*, 2006] cell lines permanently resistant to cisplatin as well as in colorectal cancer cells resistant to TRAIL [van Geelen *et al*, 2004]. Zhang *et al* (2005) showed that survivin mediates resistance to anti-androgen therapy with flutamide in prostate cancer cells. Specifically, these authors suggested that up-regulation of survivin via insulin-like growth factor-1/AKT signaling during

androgen blockade may be one of the mechanisms by which prostate cancer cells develop resistance to anti-androgens. Paik *et al.* (2004) showed that survivin was one of the sixteen genes predictive of recurrence in tamoxifen-treated breast cancer patients.

Regarding the role of survivin in determining the response of human tumour cells to radiation, Asanuma *et al.* (2000) first reported that survivin acts as a constitutive radio-resistance factor in pancreatic cancer cells. Specifically, in a panel of established cell lines they found an inverse relationship between survivin mRNA expression and *in vitro* sensitivity to X-irradiation. Moreover, they demonstrated that survivin mRNA expression was increased by sublethal doses of X-irradiation, which would suggest that the protein also acts as an inducible radioresistance factor. Rodel *et al* (2003) also showed an inverse correlation between survivin expression and apoptotic response to irradiation in a panel of colorectal carcinoma cell lines. More recently, in a translational study of 59 rectal cancer patients treated with a combination of radiotherapy and chemotherapy, the same authors reported that increased survivin expression was associated with a significantly increased risk of local tumour recurrence [Rodel *et al*, 2005]. It is worthy of note that survivin can contribute to radiation resistance also by promoting the survival of tumour vascular endothelial cells. In fact, induction of vascular endothelial apoptosis was recently shown to be a major determinant of overall tumour response to radiotherapy [Garcia-Barros *et al*, 2003]. Radiation may induce tumour cells to secrete cytokines such as vascular endothelial growth factor, which in turn could inhibit radiation-induced apoptosis of vascular endothelial cells by up-regulating survivin

expression, as already demonstrated for drug-induced apoptosis [Tran *et al*, 2002].

3. SURVIVIN DIRECTED CANCER THERAPY

In recent years considerable efforts have been made to validate survivin as a new target in cancer therapy. In this context, a collection of different approaches to counteract survivin in tumour cells, including antisense oligonucleotides, ribozymes, small interfering RNAs (siRNAs) and dominant-negative mutants, as well as cyclin-dependent kinase inhibitors, have been proposed with the dual aim to inhibit tumour growth potential and to enhance tumour cell response to apoptosis-inducing anticancer agents [Kappler *et al*, 2004; Grossmann *et al*, 1999; Andersen & Thor, 2002, Ling & Li, 2004] (Figure 8).

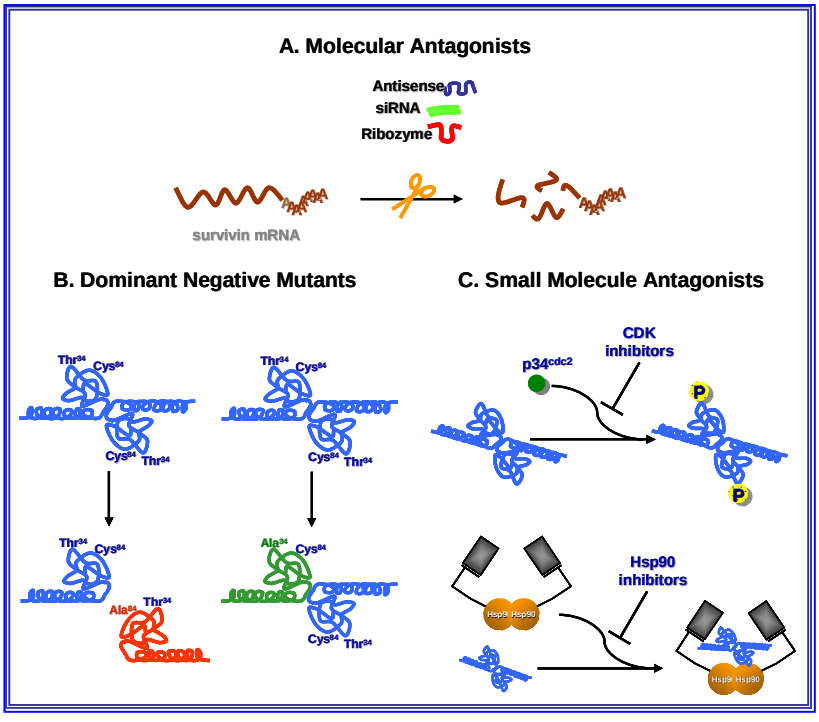

Figure 8. Summary of the current strategies for survivin targeting in cancer therapy.

A, Molecular antagonists able to target survivin mRNA and inhibit translation; **B**, Dominant negative mutants able to inhibit survivin dimerization or survivin activation; **C**, Small molecule antagonists able to inhibit survivin phosphorylation on Thr^{34} residue (CDK inhibitors) or to counteract survivin-Hsp90 interaction (Hsp90 inhibitors). In B and C, the wild type protein is reported in its dimeric arrangement [Muchmore *et al*, 2000].

Results from studies exploiting different strategies to interfere with survivin expression and function provided direct and convincing evidence that targeting the survivin network inhibits tumor growth potential in vitro and in vivo and increases spontaneous and treatment induced apoptosis of cancer cells, thus indicating survivin as a promising molecular target for cancer therapy. Two strategies, antisense oligonucleotides and small-molecule inhibitors, have entered clinical development (Table I) YM155 (Astellas Pharma) is a small-molecule survivin suppressant selected via a high-throughput screening assay with a survivin-promoter luciferase assay. It selectively inhibits survivin mRNA transcription and protein expression in several tumor cell lines and has shown potent (nmol/L) antiproliferative activity in a broad spectrum of preclinical models, including prostate, breast, ovarian, and non–small cell lung carcinomas and melanoma as well as non–Hodgkin's lymphoma and leukemia. Tumor regressions, including complete responses, have been observed in xenograft models of non–Hodgkin's lymphoma and prostate carcinoma.

In a phase I study, 41 patients were treated with a 168-hour continuous infusion of YM155 and the activity has been reported with three partial responses in non–Hodgkin's lymphoma, two prostate-specific antigen responses in hormone-refractory prostate cancer patients, and one minor response in non–small cell lung cancer [Mita *et al*, 2006].

The favorable safety profile of YM155 at the recommended dose as well as its provocative antitumor efficacy prompted the phase II evaluation of this compound in melanoma, prostate carcinoma, and non–Hodgkin's lymphoma. One of these phase II studies reported two prostate-specific antigen

responses in 32 patients with hormone-refractory prostate cancer as well as an acceptable toxicity profile for YM155 [Karavasilis *et al*, 2007]. In the melanoma study, 1 partial response and 1 minor response were observed in the 34 patients, with 2 additional patients experiencing stable disease. The non–Hodgkin's lymphoma study is ongoing, and several combination trials of YM155 with cytotoxics as well as with targeted therapies are either planned or under way.

LY2181308 (ISIS 23722; Eli Lilly and Co. and ISIS Pharmaceuticals Inc.), a secondgeneration antisense oligonucleotide targeting survivin, is also being evaluated in phase I trials.

Because survivin inhibitors have shown only modest antitumor activity in clinical trials, these agents may be best used in combination with conventional chemotherapy. Due to the complexity of the proapoptotic and antiapoptotic pathways, with multiple players involved, redundant signaling networks, and multifaceted interactions between the involved elements, blocking only one antiapoptotic factor may not result in robust antitumor activity. Therefore, the rather disappointing results of survivin inhibitors administered as a single agent in unselected populations in phase II studies are not surprising. A molecularbased enrichment of the treated populations with subjects more likely to respond to survivin inhibition could overcome, at least in part, these caveats. It was suggested that, in diffuse large-cell lymphoma, survivin may be the primary mechanism for apoptosis abrogation in a significant subset of patients, with another subset being dependent on bcl-2 overexpression and a smaller distinct population possibly relying on both [Tracey et al, 2005]. Alternatively, the combination of survivin with other

proapoptotic factors seems to be an attractive strategy with potentially broad clinical applications. There are multiple lines of evidence indicating that survivin inhibition potentializes the antitumor activity of several cytotoxics and targeted therapies, including topoisomerase inhibitors [Sato et al, 2007], alkylating agents [Li et al, 2006], tumor necrosis factora-related apoptosis-inducing ligand [Wagner et al, 2007], and UCN-01 [Vogel et al, 2007].

Overall,survivin inhibitors may represent a novel type of targeted drugs as much as they specifically interfere with defined molecular pathways of tumor cell maintenance and at the same time are applicable to different tumor types independent of their genetic makeup.

Table I. Survivin inhibitors currently in pahse I/II clinical trials.

Therapeutic Approach	Compound	Development Phase
Antisense Oligonucleotides	LY2181308 ((ISIS Pharmaceuticals)	Phase I/II
	EZN3042 (Enzon Pharmaceuticals)	Phase I
Small antisense molecule	YM155 (Astellas Pharma Inc)	Phase I/II
Antagonists of the survivin-Hsp90 complex	Shepherdin	NCI-RAID
Immunotherapy	Autologous CTL	Phase I/II

4. DNA DAMAGE

A wide diversity of lesions (Figure 9) caused by environmental agents such as ultraviolet (UV) radiation in sunlight, ionizing radiation, and numerous genotoxic chemicals can arise in the DNA. In addition, the genome is also attached by internal products of normal cellular metabolism, such as reactive oxygen species (ROS; i.e., superoxide anions, hydroxyl radicals, and hydrogen peroxide) derived from oxidative respiration and products of lipid peroxidation. These agents can cause a variety of damages in the DNA.

Cellular response to DNA-damage, during any phase of the cell cycle, is a very complex process and has the same pattern. After the detection of DNA damage by a multitude of proteins that sense the damage, signal transducer proteins transduce the signal to effector proteins into cells and execute cellular responses (Figure 9). This network of events includes a protein kinase cascade that connects the detection of DNA damage to the activation of transcription factors, which in turn regulate the expression of genes implicated in specific cellular pathways, including DNA repair mechanisms, cell-cycle checkpoints, cellular senescence and apoptosis [Zhou & Elledge, 2000; Sancar *et al*, 2004]. In response to the different types of DNA damage, the cell cycle checkpoints are activated to delay or arrest cell cycle progression, presumably in order to allow time to repair damaged DNA [Nyberg *et al*, 2002]. The blockage of DNA replication leads to collapse of replication forks and DNA double-strand breaks (DSBs) formation, which are thought to be crucial downstream apoptosis triggering lesions [Roos & Kaina, 2006]. DSBs are recognised by proteins that exert both kinase and repair

activity. The most important players in the early response to DNA damage consists of two kinases, members of the phosphatidylinositol-3-kinase-related family [Durocher & Jackson, 2001; Ismail *et al*, 2005]: ATM (Ataxia Telangiectasia Mutated) and ATR (Ataxia Telangiectasia and Rad3 related). Ataxia telangiectasia is an autosomal recessive disorder caused by mutated ATM, characterized by immunodeficiency, neurological disorders, and high cancer susceptibility. ATR was identified later on basis of sequence and functional homology to ATM. These kinases phosphorylate p53 at serine 15 in response to DNA damage, resulting in the stabilization of the protein and subsequently amplifying the downstream p53 cascade and obtaining p21 blocking the cell cycle [Siliciano *et al*, 1997]. The ATM and ATR proteins belong to the phosphatidylinositol 3-kinase like (PIKK) family of serine/threonine protein kinases. This family also includes DNA protein kinase (DNA-PK). DNA-PK has an important role in G1/S checkpoint and seems to be a DNA double strand break repair enzyme [Durocher & Jackson, 2001].

Recent results suggest that the Rad50/Mre11/Nijmegen Breakage Syndrome 1 (NBS1) complex and Rad17 protein function as the DSBs sensor for ATM [Williams, 2007]. Whereas ATM is activated by ionizing radiation-induced DSBs, ATR is activated in response to UV light and presumably all chemical agents that give rise to stalled DNA replication forks [Dart *et al*, 2004]. Other candidate DNA damage sensors are three proteins Rad9, Hus1, and Rad1 that form a ring structure (the so called “9–1–1” complex) that can encircle the damaged DNA and is expected to form a

scaffold for downstream checkpoint and repair proteins [Houtgraaf *et al*, 2006].

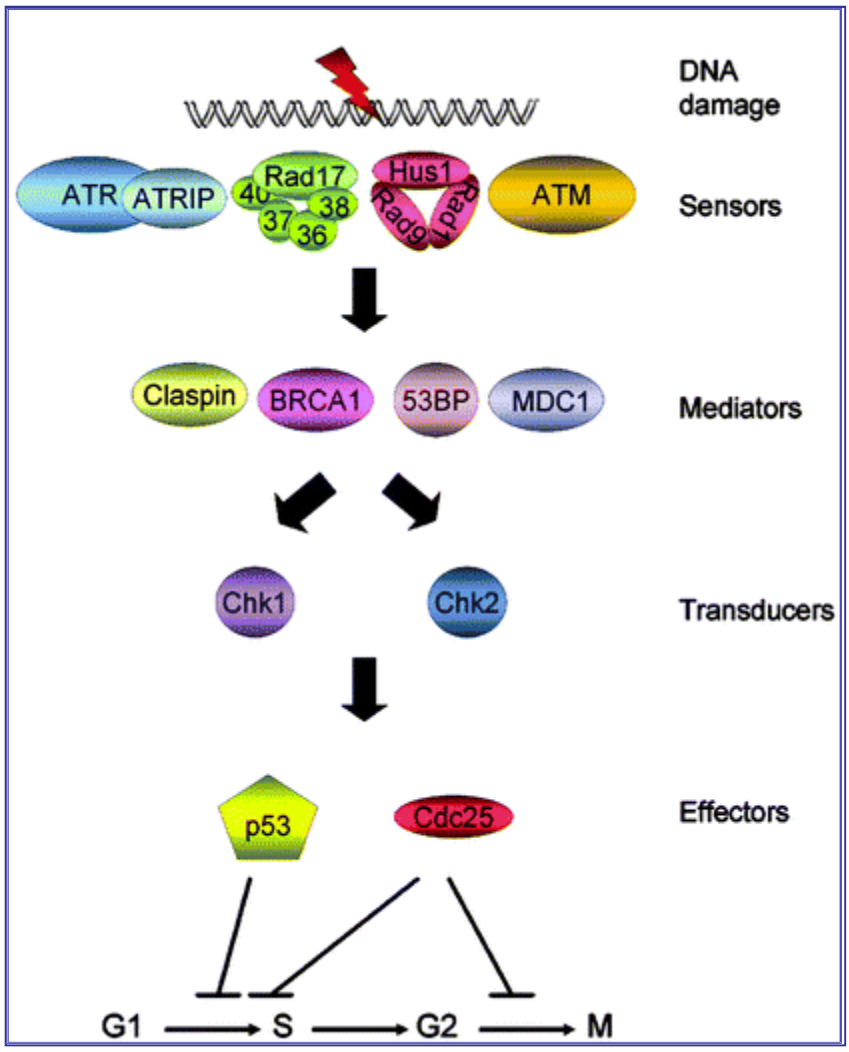

Figure 9. Representation of the DNA-damage-induced checkpoint response.

After the detection of a given damage by sensor proteins, this signal is transduced to the effector proteins via the transducer proteins. Depending on the phase of the cell cycle in which the cell is, this can lead to activation of p53 and inactivation of CDC25, which eventually leads to cell cycle arrest.

Upon activation, ATM phosphorylates various downstream substrates such as p53, Nbs1, MDC1, 53BP1, Rad9, Chk1 and Chk2, H2AX and BRCA1 [Lavin *et al*, 2005; Roos & Kaina, 2006]. ATM, and presumably also ATR, exerts three crucial functions: regulation and stimulation of DSB repair, signaling cell-cycle checkpoints and signaling apoptosis via p53 [Lavin *et al*, 2005]. Once phosphorylated, p53 becomes stabilised and blocks proliferation by up-regulation of $p21^{waf1}$, which triggers G_1/S arrest. It has been suggested that at low levels of DSBs only a minor fraction of p53 is activated, driving the transcription of the *p21* gene and causing cell-cycle arrest. By contrast, with high levels of DSBs, p53 becomes metabolically stable and transcriptionally activated, and accumulates above a particular threshold, inducing the transcriptional activation of pro-apoptotic genes such as Bax (Bcl-2-associated X protein), PUMA (p53 up-regulated modulator of apoptosis) and FAS receptor [Roos & Kaina, 2006]. p53 activation results from post-translational modifications (i.e., phosphorylations, acetylations or covalent attachments of small ubiquitin-like proteins), whereas p53 instability is due to its ubiquitination and proteosomal degradation. Both these processes are transiently suppressed after DNA damage [Bernstein *et al*, 2002].

Most human tumours lose the expression of a functional p53. However, it has been demonstrated that crucial DNA damage can activate a p53-independent apoptosis via backup systems [Roos & Kaina, 2006]. There are several strategies that cells seem to employ to trigger p53-independent DNA damage-induced apoptosis. The first one involves the p53 homologs p63 and p73 [Roos & Kaina, 2006]. It has been proposed that, upon DNA damage, ATM and ATR activate Chk1 and Chk2, which are like ATM and ATR,

serine/threonine protein kinases and phosphorylate targets that eventually result in the cell cycle arrest.

The Double Strands Breaks (DSBs) signal sensed by ATM is transduced by Chk2, and the UV damage signal sensed by ATR is transduced by Chk1, although there is some overlap and redundancy between the functions of these two proteins. Chk1 and Chk2 transfer the signal of DNA damage to the phosphotyrosine phosphatases and cell division cycle proteins Cdc25A, Cdc25B, and Cdc25C. Phosphorylation of Cdc25A–C by Chk1 or Chk2 inactivates Cdc25A–C, whereas unphosphorylated Cdc25A–C promotes the G1/S and G2/M transition by dephosphorylating the cyclin dependent kinases (CDKs) directly involved in cell cycle transition.

The exact pathway of cell cycle arrest depends on the kind of damage. DNA DSBs lead to phosphorylation of ATM that subsequently phosphorylates Chk2. Single-strand gaps result in the activation of Rad17 or the 9–1–1 complex, and ATR, which leads to phosphorylation of Chk1. Subsequent phosphorylation of Cdc25A by Chk1 or Chk2 causes inactivation of this protein by nuclear exclusion and ubiquitin-mediated proteolytic degradation, leading to G1 arrest. ATM and ATR also phosphorylate p53, which leads to stabilization and accumulation of the p53protein and promotes its transcription factor activity. Two pathways mediate, instead, the intra-S-phase checkpoint. Firstly, the ATM/ATR–Chk2/Chk1– Cdc25A–CDK2 pathway is more or less similar to the G1/S checkpoint. In the S phase, this pathway delays replication (by blocking the loading of Cdc45 onto chromatin that in turn attracts DNA polymerase-a into prereplication complexes) and, as a consequence, extends the DNA replication time, allowing DNA repair to

take place. The second pathway involves Nbs1 which is phosphorylated by ATM together with Chk2, leading to a cascade involving also Mre11- and Rad50-like initial DSBs recognition, which plays a role not only in cell cycle arrest but also in activating the repair processes. When cells encounter DNA damage in G2, the G2/M checkpoint stops the cell cycle in order to prevent the cell from entering mitosis. As in the G1/S checkpoint, the kind of DNA damage determines the pathway that will be activated: ATM–Chk2–Cdc25 for DSBs and ATR–Chk1– Cdc25 for DNA lesions such as those created by UV light. Besides downregulating Cdc25A, both Chk1 and Chk2 upregulate WEE1 by phosphorylation, which together control Cdc2/CyclinB activity. This latter complex promotes G2/M transition under normal circumstances, and inactivation blocks the cell cycle when damage occurs in G2.

Activation of Chk1 and Chk2 by ATM and ATR in turn activate E2F1. This in turn stimulates transcription of the *p73* gene, giving rise to an increased level of p73 protein [Urist *et al*, 2004]. Whereas p53 requires p63 and p73 for triggering apoptosis, p73 has a pro-apoptotic activity even in the absence of p53 [Flores *et al*, 2002; Roos & Kaina, 2006]. p73-induced apoptosis was shown to be mediated by transcriptional up-regulation of PUMA, NOXA and Bax [Melino *et al*, 2004; Flinterman *et al*, 2005].

Another factor implicated in p53-independent apoptosis is NF-kB (nuclear factor-kB), which is generally anti-apoptotic as it transcribes anti-apoptotic genes. However, under some circumstances (i.e., topoisomerase inhibitor treatment [Piret *et al*, 1999], NF-kB exhibits pro-damage-signaled apoptosis, by inducing the transcription of a growing number of genes related to apoptosis, including Fas ligand [Schmitt *et al*, 2007]. In addition, upon DNA

damage RNA synthesis is inhibited, leading to a decline in the level of critical gene products such as MKP1 (mitogen-activated protein kinase phosphatase) and causing sustained activation of JNK (Jun kinase) and AP-1, which stimulates death-receptor activation [Roos & Kaina, 2006].

5. DNA REPAIR PATHWAYS

The DNA damage has not only to be prevented, but also to be repaired when occurred. As there are many different lesions possible, different types of repair pathways have evolved. Important pathways in mammalian cells include base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and DSBs repair (Figure 10).

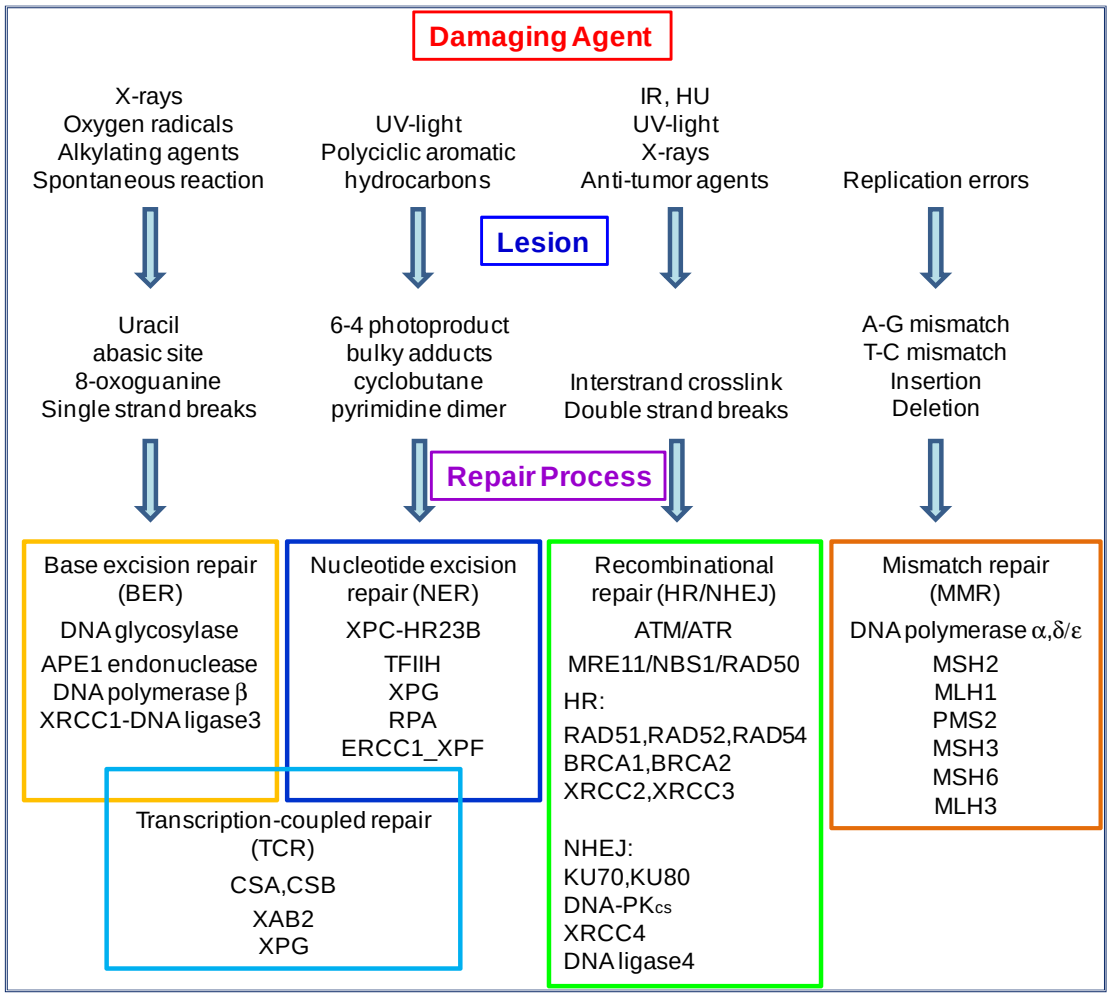

Figure 10. Summary of the most common types of DNA lesions that can be caused by exogenous or endogenous damaging agents.

5.1. DNA base excision repair

The DNA base excision repair (BER) pathway plays a critical role in maintaining genetic stability and is is the main guardian against damage due to cellular metabolism. BER is responsible for correcting base lesions arising from damage due to oxidation, alkylation, deamination and depurination / depyrimidination [Xu *et al*, 2008; Robertson *et al*, 2009].

Base damages are generated by ROS, ionizing radiation, and indirectly also by UV radiation (via generation of ROS) or can be the result of various chemicals like chemotherapeutic drugs (e.g., adriamycin, mitomycin C, and psoralen). BER encompasses two general subcategories; short-patch BER involves repair of a single nucleotide while long-patch BER produces a repair tract of at least two nucleotides. There are four general steps to BER, first a glycosylase (depending on the damage) recognizes and catalyzes the removal of the damaged base(s). Next a nuclease (APE1 endonuclease) causes strand incision. This results in an abasic site, from which both ends are trimmed by poly(ADP-ribose) polymerase and polynucleotide kinase to facilitate repair synthesis. Finally a ligase seals the broken DNA strand (Figure 11). Genetic mutations in BER components have been linked to familial colorectal cancer [Al-Tassan *et al*, 2002; Jones *et al,* 2002; Sampson *et al*, 2003; Sieber *et al*, 2003; Croitoru *et al*, 2004; Isidro *et al*, 2004; Venesio *et al*, 2004; Wang *et al*, 2004; Eliason *et al*, 2005].

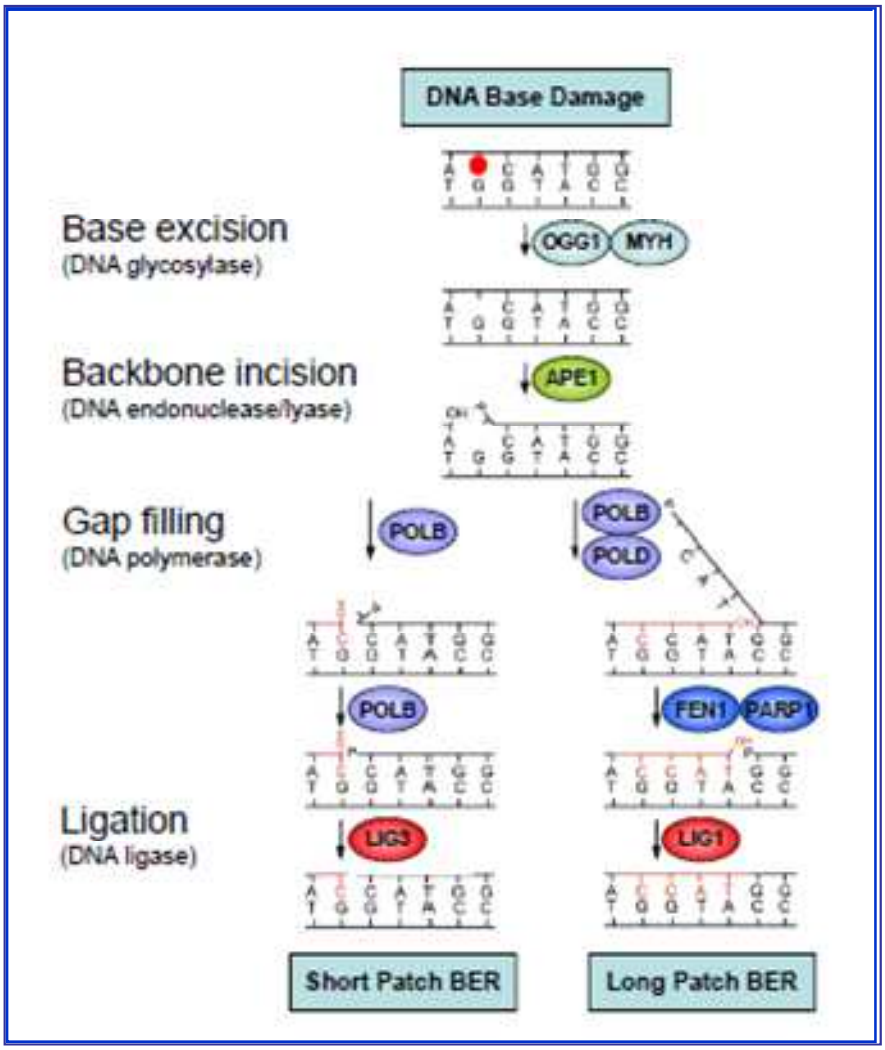

Figure 11. Simplified schematic of DNA base excision repair.

The base excision repair (BER) pathway plays a critical role in maintaining genetic stability and is responsible for correcting base lesions arising due to oxidation, alkylation, deamination and depurination / depyrimidination. There are four general steps to BER, first a glycosylase recognizes and catalyzes the removal of the damaged bases. Next a nuclease causes strand incision, followed by gap-filling by a polymerase. Finally a ligase seals the broken DNA strand. Replacement of a single nucleotide is termed short patch BER while replacement of 2-6 nucleotides is termed long patch BER. (Adapted from Robertson et al.; Cellular and Molecular Life Sciences, 2009.)

5.2. Nucleotide excision repair

NER is the most important repair system to remove damage that distorts the normal architecture of the DNA helix and bulky DNA lesions that can be caused by UV radiation (thymidine dimers), chemicals, or ROS. Disruption of the DNA helix interferes with base pairing and hinders transcription and normal replication. The repair of damaged DNA involves at least 30 polypeptides within two different sub-pathways of NER known as transcription-coupled repair (TCR-NER) and global genome repair (GGR-NER) [Reardon & Sancar, 2005]. Repair is much more efficient in actively transcribed genes than in the overall genome, and the two pathways differ only in the initial DNA damage recognition step. NER includes a lot of different proteins but the most important of them are encoded by seven xeroderma pigmentosum (XP) complementation groups, XPA to XPG genes.

5.3. DNA mismatch repair

DNA mismatch repair (MMR) is responsible for recognizing and repairing the erroneous insertion, deletion and mis-incorporation of bases that arise as a result of replication errors that escape the proofreading function of DNA polymerases [Harfe & Jinks-Robertson, 2000; Hsieh & Yamane, 2008]. In eukaryotes, MMR begins with the recognition of the mismatch by the MutS complex. MutS then works in concert with the MutL complex to facilitate

endonucleolytic cleavage by a third MMR protein, PMS2. Single strand exonucleases resect the DNA allowing for DNA POLD and Ligase I to repair the resulting DNA gap [Hsieh & Yamane, 2008]. Consequently, loss of functional MMR leads to microsatellite instability (MSI) and a mutator phenotype [Wu *et al*, 2000; Umar *et al*, 2004].

5.4. DNA double strand break repair

The described repair systems are efficient only with damaging agents that cause single strand lesions but the most dangerous and critical DNA damages that occurs on DNA are the double strand breaks (DSBs) . DSBs are a very genotoxic type of DNA damage which are primarily repaired by the Homologous Recombination (HR) or Non Homologous End Joining (NHEJ) pathways in mammalian cells (Figure 12 and 13) [Rothkamm *et al*, 2003; Helleday *et al*, 2007]. Because both strands of the DNA double helix are broken, chromosomal fragmentation, translocation, and deletions can easily occur and rapid repair is crucial. DNA DSBs can be caused by ionizing radiation, ROS, and chemotherapeutic drugs and can arise during replication of a single-strand break. The two main differences between these pathways are the requirement for extensive DNA homology on the sister chromatid in HR and the accuracy of repair.

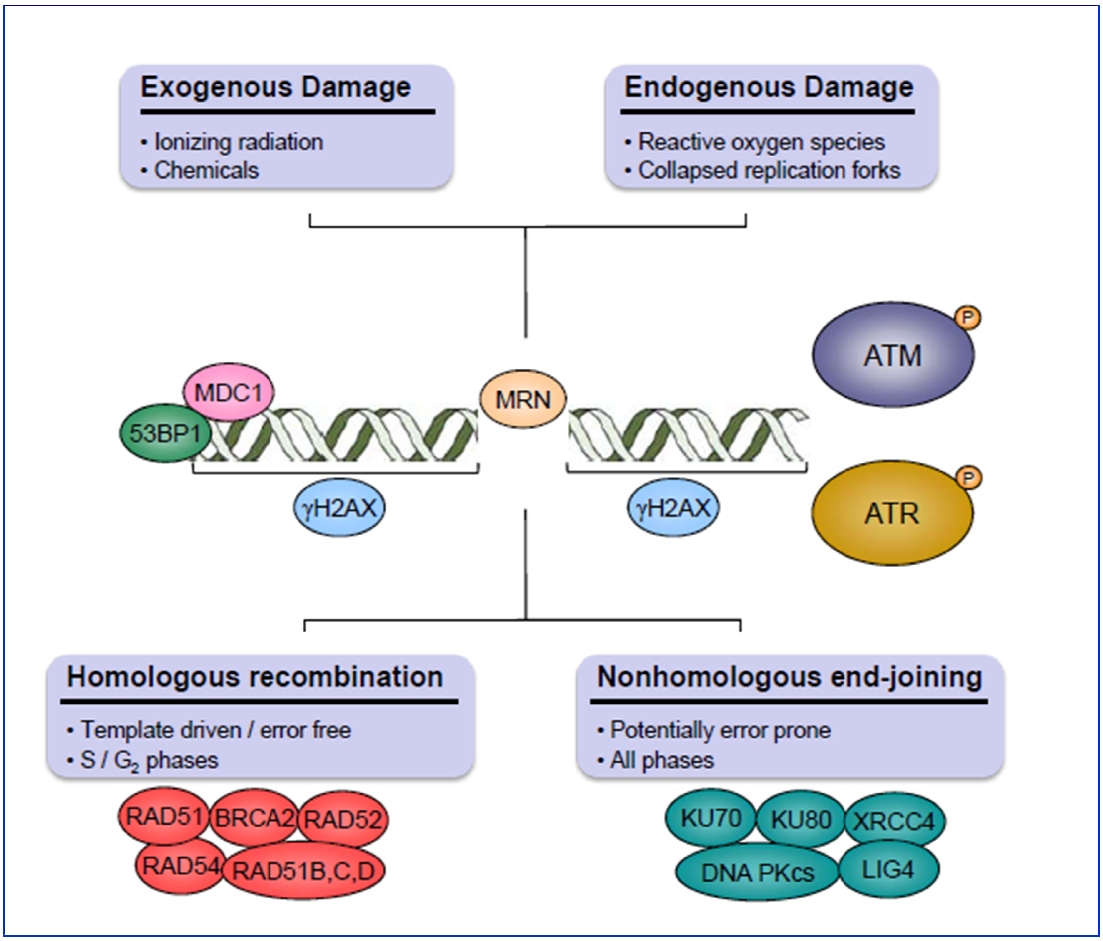

Figure 12. DNA double strand break sensing and repair in human cells.

Under normal conditions, a DNA double strand break (DSB) is sensed by the MRE11–RAD50–NBS1 (MRN) complex. This leads to activation and recruitment of the ATM and DNA-PKCS kinases and phosphorylation of the histone variant H2AX (termed γH2AX) around the site of the break. Subsequently, a number of DNA damage sensing proteins (such as MDC1 and 53BP1) and DNA DSB repair proteins involved in homologous recombination (HR) and nonhomologous end joining (NHEJ) are recruited within the first 1–6 h of damage recognition to repair the DNA DSB. The NHEJ repair pathway can be used within any phase of the cell cycle and can be error-prone. The HR pathway is preferentially active in the S and G2 phases of the cell cycle when a homologous sister chromosome or chromatid is available for direct base-pairing to effect error-free repair of a DNA DSB. (Adapted from Bristow and Hill; Nature Cancer Reviews, 2008.)

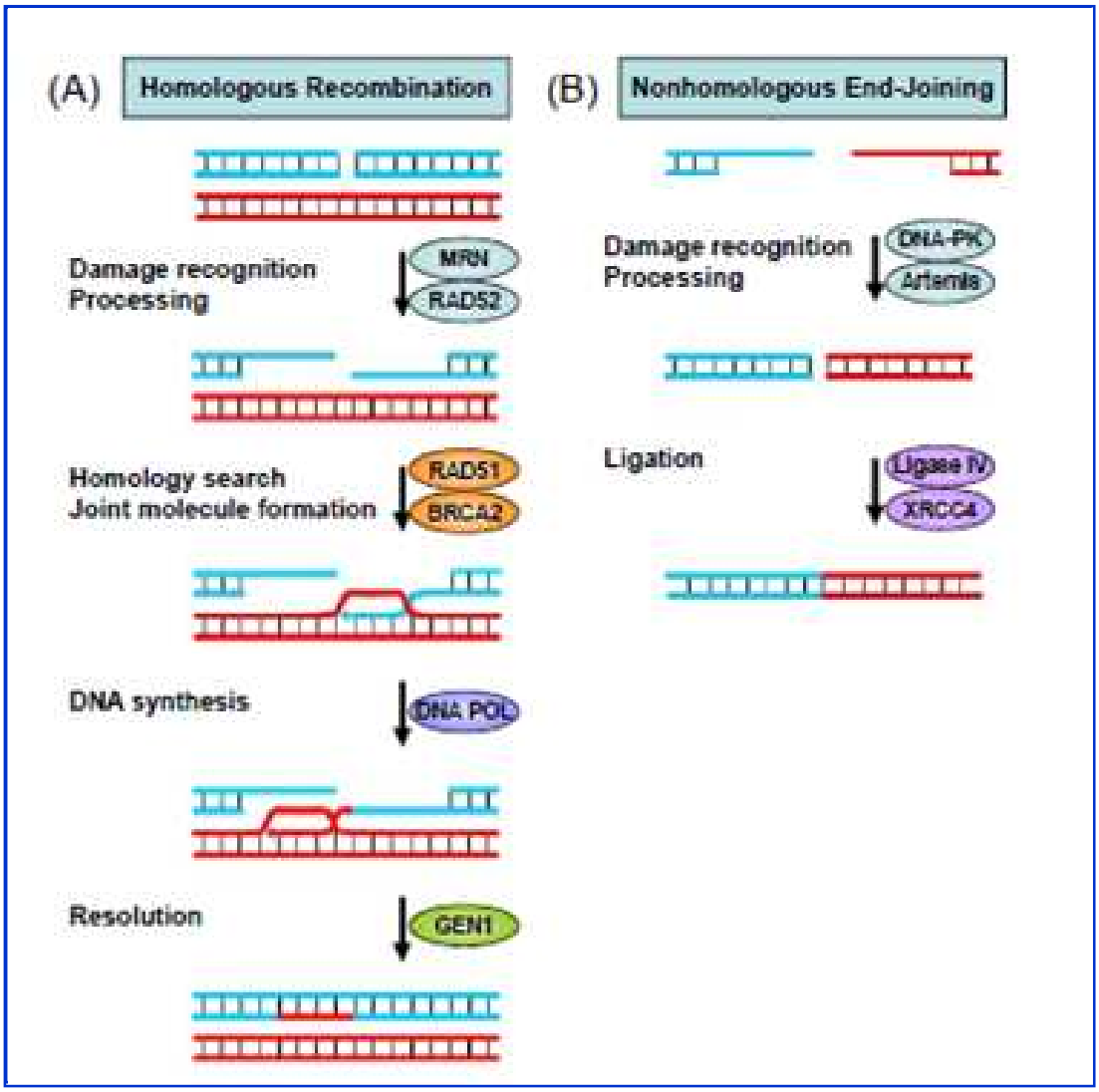

Figure 13. Simplified schematic of DNA double strand break repair in mammalian cells.

A. Homologous recombination (HR) uses a homologous DNA fragment as a template for accurate repair. Free DNA ends at the site of a DSB are first processed followed by association of Rad51, Rad52 and RPA with the single-stranded overhangs. This nucleoprotein filament searches for homologous DNA and subsequently, a joint molecule is formed allowing for template guided DNA synthesis.
B. Nonhomologous end-joining (NHEJ) does not require the presence of a homologous template, but mediates repair by directly rejoining DNA strands. It involves recognition of the DSB, processing of non-complementary or damaged DNA ends and the subsequent ligation of the DNA termini. Processing of DNA ends can lead to loss or gain of nucleotides, rendering NHEJ less accurate than HR. (Adapted from Weterings et al.; DNA Repair, 2004.)

DSBs are initially sensed by the MRE11–RAD50–NBS1 (MRN) complex. This leads to activation and recruitment of the ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK) catalytic subunit (DNA-PKCS) kinases and phosphorylation of the histone variant H2AX (termed γH2AX) around the site of the break. Subsequently, a number of DNA damage sensing proteins, such as mediator of DNA damage checkpoint 1 (MDC1) and p53-binding protein 1 (53BP1), and DNA DSB repair proteins involved in HR and NHEJ are recruited within the first 1–6 h of damage recognition to repair the DNA DSB. HR is a template-guided, error-free repair pathway, predominantly operating in the S and G2 phases of the cell cycle as it requires a repair template from a sister chromatid or chromosome [Rodrigue *et al*, 2006; Helleday *et al*, 2007; Ip *et al*, 2008]. Free DNA ends formed at the site of a DSB are first processed to produce single stranded DNA (ssDNA) with a 3'-hydroxyl overhang involving the action of the MRN complex along with the CtIP, Exo1, BLM and BRCA1 proteins [Ira *et al*, 2003; Lengsfeld *et al*, 2007; Mimitou *et al*, 2008; Yun & Hiom, 2009]. Replication protein A (RPA) binds and stabilizes the resulting ssDNA [Sung & Klein, 2006]. RAD51 displaces RPA to form the nucleoprotein filament with the assistance of BRCA2 [Sharan *et al*, 1997; Wong *et al*, 1997; Pellegrini *et al*, 2002]. This nucleoprotein filament subsequently searches for homologous DNA and when found facilitates the formation of a D-loop intermediate in which the 3'-end initiates DNA synthesis using the duplex DNA as a template [Weterings & van Gent, 2004].

The joint DNA molecule (termed a Holliday junction) can be resolved by the Holiday junction resolvase, GEN1 [Ip *et al*, 2008] or the BLM-

topoisomerase IIIα [Wu & Hickson, 2003] or Mus81-Eme1 complexes [Chen *et al*, 2001; Constantinou *et al*, 2002].

In contrast, NHEJ is predominant in G1, but can act throughout the cell cycle as it does not require a homologous template for repair. The two ends of the broken double helix are directly ligated together by the DNA ligaseIV/Xrcc4 complex. It involves recognition of the DSB, processing of noncomplementary or damaged DNA ends and the subsequent ligation of DNA termini. Processing of DNA ends can lead to loss or gain of nucleotides, rendering NHEJ less accurate than HR [Weterings & van Gent, 2004; Helleday *et al*, 2007]. Other proteins involved in this pathway are the Ku70/80 heterodimer, DNA-PKCS, and the Rad50/Mre11/NBS1 complex.

Although both DSB repair pathways are operational in mammals, their relative contribution differs depending on the stage of the cell cycle or the cell type. For HR to occur, there is a need for a sister chromatid, which is not produced until the S phase. For this reason, HR can only take place in dividing cells that are in the S or G2 phase. Cells in G0 and G1 or terminally differentiated cells mainly rely on NHEJ. A number of the previously mentioned DNA DSB repair proteins (for instance, Rad51, Rad54, and the MRN complex) and γ-H2AX relocate into nuclear foci after induction of DNA damage. These foci are rapidly formed. But what is their function? There are different hypotheses about that. One of these is that the efficiency of repair and signaling may be enhanced by the concentration of factors near the lesion that would generate amplification of signals [Celeste *et al*, 2003]. Histone H2AX may function to concentrate proteins near DNA lesions. A second hypothesis considers that phosphorylated residues on H2AX may

serve as docking sites for the assembly of proteins in chromatin regions distal to the breaks. Therefore, γ-H2AX could be involved in the retention of repair/signal factors in the vicinity of the lesion [Bassing *et al*, 2002].

The retention and subsequent increase in the local concentration of factors may be mediated, in particular, via weak interactions between the high-phosphorylated H2AX tail, and specific domains of repair/signaling proteins, while the changes in chromatin structure can promote intermolecular auto-phosphorylation of ATM even at a distance [Bakkenist & Kastan, 2003]. It is still object of debate whether ATM directly senses these DSB-induced chromatin alterations or requires specific damage alerting ‘‘sensors’’. In any case, it is ascertained that H2AX phosphorylation determines both chromatin condensation and amassing of repair factors at the site of damage. This focal assembly of repair factors complex and associated changes in chromatin folding facilitates the juxtaposition of broken ends [Ward & Chen, 2001]. At this point, the DNA repair may take place. However, this repair is not an easy issue.

6. CHECKPOINT KINASE 2

Checkpoint kinase 2 (Chk2) is a multifunctional enzyme whose functions are central to the induction of cell cycle arrest and apoptosis by DNA damage [Ahn *et al*, 2004]. Insight into Chk2 has derived from multiple approaches. Biochemical studies have addressed Chk2 structure, domain organization and regulation by phosphorylation. Extensive work has been done to identify factors that recognize and respond to DNA damage in order to activate Chk2. In turn a number of substrates and targets of Chk2 have been identified that play roles in the checkpoint response. The roles and regulation of Chk2 have been elucidated by studies in model genetic systems extending from worms and flies to mice and humans. The relationship of Chk2 to human cancer studies is developing rapidly with increasing evidence that Chk2 plays a role in tumor suppression.

6.1. Domains of Chk2

The Chk2 protein consists of three distinct functional domains; an SQ/TQ cluster domain (SCD, residues19–69), a forkhead associated domain (FHA, residues112–175), and a Ser/Thr kinase domain (residues 220–486) (Figure 14a). The SCD contains five SQ and two TQ motifs, which satisfy the primary substrate motif for ataxia-telangectasia mutated (ATM) kinase [Kim *et al,* 1999]. Especially residues 67–70, STQE, are closely related to the refined substrate motif (LSQE) of ATM kinase, determined by oriented peptide library

search [O'Neill *et al*, 2000], and T68 was identified to be a key phosphorylation site [Bartek *et al*, 2001; Kastan & Bartek, 2004]. The FHA domain is an 80–100 amino acid phosphopeptide binding domain, conserved from yeast to human [Li *et al*, 2000].

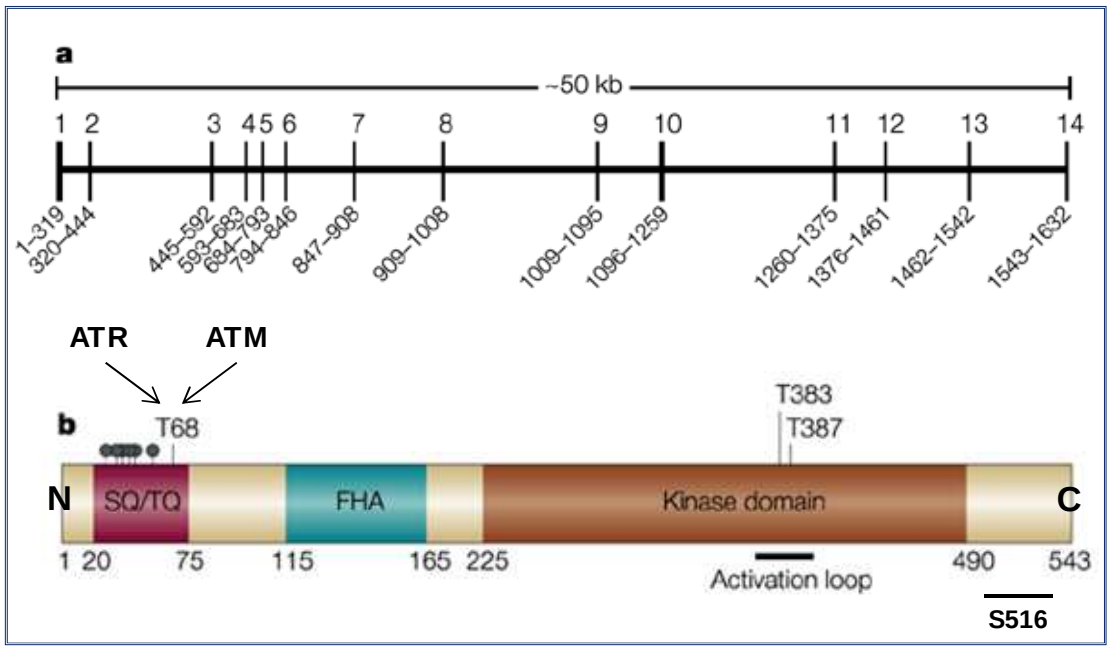

Figure 14. Structure of the human *CHK2* gene and CHK2 protein.

a) The *CHK2* gene localizes to chromosome 22q12.1, which spans approximately 50 kilobases (kb), and consists of 14 exons (black boxes). Numbers below the bars indicate the base-pair (bp) range of each exon. Highly homologous fragments of the gene that include exons 11 to 14 are found on chromosomes 2, 7, 10, 13, 15, 16, X and Y.
b) The SQ/TQ-rich, forkhead-associated (FHA) and kinase domains are shown in maroon, blue and brown, respectively. Pinheads indicate putative ATM/ATR phosphorylation sites in the SQ/TQ-rich region. In vivo phosphorylations of CHK2 on threonine 68 (by ATM/ATR) and threonines 383 and 387 in the activation loop (autocatalytic) are indicated. (Adapted from Bartek et al.; Nature Reviews Molecular Cell Biology,2001)

The domain consists of 11 beta sheets forming a structural core and connecting loops conferring diversity in phosphopeptide recognition as resolved by structural analyses of several FHA domains with and without

phosphopeptides by X-ray crystallography [Durocher *et al,* 1999; Durocher *et al*, 2000; Li *et al,* 2002; Stavridi *et al,* 2002] and nuclear magnetic resonance (NMR) spectroscopy [Liao *et al*, 1999; Liao *et al,* 2000; Wang *et al*, 2000; Byeon *et al*, 2001; Yuan et al, 2001]. FHA domains are found in proteins involved not only in DNA damage checkpoint pathways, but also kinesin family members, transcription factors, Ki-67, and nuclear inhibitors of protein phosphatase 1 [Durocher & Jackson, 2002]. The FHA domain functions in *trans* to modulate protein–protein interactions but also in *cis* to affect other functional domains within the protein itself. The kinase domain shares homology to other Ser/Thr or Tyr kinases with a Gly-rich region at the vicinity of Lys residues in the N-terminal part and Asp as a catalytic residue at the active site.

6.2. Chk2 & Cancer

Chk2 has established its position as a bona-fide tumor suppressor and the list of Chk2 mutations in cancer cells is continuously expanding [Bartek & Lukas, 2003]. The first indication of a tumor suppressor function for Chk2 came from the finding that a sub-population of families with the Li-Fraumeni syndrome (LFS), a familial cancer predisposition syndrome usually associated with germline TP53 mutation [Evans & Lozano, 1997], contain *wild type* p53 but mutant Chk2 suggesting that germline mutations in the CHEK2 gene phenocopy TP53 inactivation [Bell *et al,* 1999]. The CHEK2

allele containing the mutation 1100delC encodes a C-terminally truncated protein within the kinase domain and confers increased breast cancer risk independent of mutation in the breast cancer susceptibility associated (BRCA) loci [Vahteristo *et al*, 2002; Meijers-Heijboer *et al*, 2002; Meijers-Heijboer *et al*, 2003]. Screening of cancer cell lines has revealed missense mutations in all three functional domains in a variety of tumor types [Sodha *et al*, 2002; Schutte *et al*, 2003; Dong *et al*, 2003]. The functional impact of CHEK2 mutation generally reflects its location. Thus, kinase domain mutants lead to faulty kinase activation after ionizing radiation [Wu *et al,* 2001], while FHA domain lesions may accelerate Chk2 degradation [Lee *et al,* 2001; Matsuoka *et al*, 2001], or may induce structural changes conferring defects in protein–protein interaction [Li *et al*, 2002; Falck *et al*, 2001a, Falck *et al*, 2001b]. It will be of interest to determine at what stage of tumor development CHEK2 mutations arise and the precise Chk2 functions that are selected against.

6.3. Regulation & Activation of Chk2

Chk2 kinase activity is increased following DNA damage induced by ionizing radiation, chemotherapeutic agents, or other compounds that harm DNA directly or indirectly. Recently, senescence triggered by telomere erosion has been implicated as an activating signal for Chk2 [d'Adda di Fagagna *et al,* 2003; Oh *et al*, 2003]. Signals from damaged DNA are transmitted to the ATM kinase, which has a number of substrates involved in

the checkpoint cascade [Banin *et al,* 1998; Canman *et al*, 1998; Shiloh, 2003]. Multiple lines of evidence establish a strong link between ATM and Chk2. First, Chk2 fails to be activated in A–T cells and restoration of a Chk2-dependent S-phase checkpoint was observed upon ectopic expression of ATM in A–T cells [Matsuoka *et al,* 1998; Brown *et al,* 1999; Chaturvedi *et al*, 1999]. Second, ATM directly phosphorylates Chk2 at Thr68, a consensus site in the SCD domain in vitro [Ahn *et al,* 2000; Melchionna *et al*, 2000] and alanine mutation of this residue prevents activation of Chk2 by gamma irradiation in vivo [Ahn *et al,* 2000; Matsuoka *et al*, 2000; Melchionna *et al*, 2000]. The Ataxia-Telangectasia-and-Rad3-related (ATR) kinase may similarly phosphorylate Chk2 after high levels of DNA damage or other stressors such as ultraviolet radiation (UV) or treatment with the ribonucleotide reductase inhibitor Hydroxyurea (HU) [Matsuoka *et al,* 2000]. Chk2 exists as a monomer in unperturbed cells. Following DNA damage, Chk2 undergoes dimerization in which a region spanning phosphorylated Thr68 in one Chk2 molecule binds to the FHA domain of second molecule [Ahn *et al*, 2002; Xu et al, 2002; Ahn & Prives, 2002]. Dimerization is likely followed by multiple intermolecular phosphorylation events including Thr383 and Thr387 within the auto-inhibitory loop resulting in kinase activation (Figure 15) [Lee & Chung, 2001]. Autophosphorylation of Ser516 within the kinase domain was also recently identified, and is required for full kinase activation [Schwarz *et al,* 2003; Wu & Chen, 2003]. Furthermore, mutation of this residue prevents radiation-sensitization by Chk2, suggesting a functional role for autophosphorylation [Wu & Chen, 2003]. Phosphorylation at Thr68 is likely to be the initiating event for dimerization and activation since

maintenance of Thr68 phosphorylation is not required for sustained kinase activity [Ahn & Prives, 2002]. Phosphorylation of other sites are likely to mediate stress specific differences in the extent of kinase activation. Indeed, Chk2 purified after gamma radiation, UV, and HU treatment showed different kinase activities toward Cdc25C, one of its well validated substrates [Matsuoka *et al*, 1998]. Moreover, the phospho-Thr68 to FHA domain intramolecular interaction mechanism may also function in stress induced association with other checkpoint proteins. For example, the MDC1 checkpoint protein FHA domain binds selectively to Chk2 phosphorylated at Thr68 [Lou *et al,* 2003]. The Polo Like Kinase 1 (PLK1), a centrosome-associated kinase implicated in mitotic progression, can interact with Chk2 and colocalize with Chk2 at centrosomes. Interestingly, PLK1 can phosphorylate residues in the N-terminus of Chk2 including Thr68 [Tsvetkov *et al*, 2003]. The functional import of this interaction for PLK1 or Chk2 function is not known. The related kinase PLK3 also interacts with Chk2 and phosphorylation of Chk2 and PLK3 by one another has been reported [Xie *et al,* 2002, Bahassi *et al*, 2002]. The subcelluar localization of Chk2 may be regulated by another recently described binding protein, karyopherin–alpha2 [Zannini *et al*, 2003].

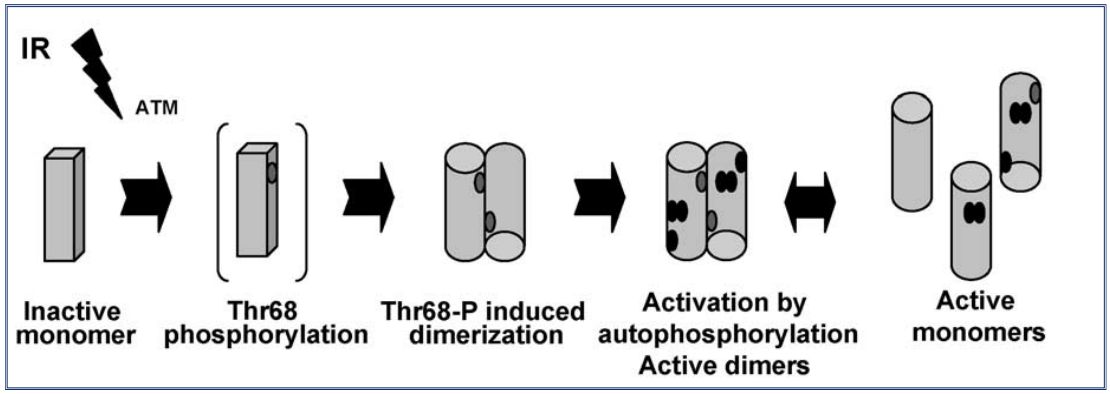

Figure 15. Model of Chk2 Activation.

A model for gamma irradiation induced formation of active dimmers and monomers. After phosphorylation of inactive monomers at T68 by ATM, dimers form and undergo subsequent autophosphorylation and may also serve as substrates for other protein kinases. Phosphorylated dimers or monomers are active in phosphorylating substrates such as Cdc25C. (Adapted from Ahn *et al.*; DNA Repair, 2004)

6.4. Substrates of Chk2

Upon activation Chk2 relays the checkpoint activation signal to a number of effectors, which mediate many of the phenotypic characteristics provoked by DNA damage including cell cycle arrest and apoptosis (Figure 16). Use of oriented peptide libraries has shown Chk2 preferentially phosphorylates a consensus motif, L-X-R-X-X-S/T, [O'Neill *et al,* 2002; Seo *et al*, 2003]. However, these studies may only describe one aspect of Chk2 kinase specificity as they do not capture the in vivo complexity of kinase-substrate interaction.

Nevertheless, many functionally important substrates bearing this motif have been identified. The first described and best validated of Chk2 substrates are two members of the cell division cycle 25 dual specificity

phosphatase family, Cdc25A and Cdc25C. The Cdc25 family promotes cell cycle progression by activation of the cyclin-dependent kinases cdk2 and cdk1 through dephosphorylation of inhibitory phosphorylation at Thr14 and Tyr15 [Matsuoka *et al*, 1998; Falck *et al*, 2001; Brown *et al*, 1999; Blasina *et al*, 1999]. Cdc25A is destabilized by Chk2 phosphorylation at Ser123 [Falck *et al,* 2001; Sorensen *et al*, 2003] most likely by increasing susceptibility to the SCF_TCRP complex, which mediates Cdc25A turnover [Busino *et al*, 2003; Jin et al, 2003]. Chk2 phosphorylation of Ser216 in Cdc25C creates a binding site for 14-3-3 proteins [Zhou *et al*, 2000] and this interaction is thought to result in persistent cytoplasmic Cdc25C localization thus preventing the G2/M transition as the cdk1/Cyclin B complex cannot be activated [Peng *et al*, 1997; Dalal *et al*, 1999]. Taken together, phosphorylation of Cdc25A and Cdc25C by Chk2 in response to DNA damage explains in part how the checkpoint induces cell cycle arrest.

Chk2 phosphorylates E2F-1 at Ser364 in response to the DNA-damaging agent etoposide, and the modification of that residue regulates both E2F-1 stabilization and transcriptional activity [Stevens *et al,* 2003]. Given the observation that E2F-1 null thymocytes are resistant to etoposide-induced apoptosis [Lin *et al*, 2001] and that over-expression of E2F-1 can induce cell death [DeGregori *et al*, 1997], modulation of E2F-1 activity represents a potentially important pathway through which Chk2 may regulate DNA damage-induced apoptosis.

Two checkpoint pathway scaffold proteins, BRCA1 and Promyelocytic Leukemia (PML), can interact with Chk2 and may be phosphorylated by Chk2 following DNA damage [Lee *et al*, 2000; Yang *et al*, 2002].

Interestingly, both substrates follow a similar pattern in which Chk2 interacts with PML and BRCA1 in the absence of DNA damage but following gamma irradiation BRCA is phosphorylated at Ser988 and PML is phosphorylated at Ser117 in a Chk2-dependent fashion, resulting in subsequent inhibition of binding with Chk2. Alanine mutation of either phosphorylation site prevents release of Chk2 from BRCA1 or PML after DNA damage suggesting a relationship between activation of phosphorylation and inhibition of interaction. Functionally, BRCA1Ser988A fails to increase survival of BRCA1-null HCC1937 cells following IR and BRCA1Ser988A stably expressed in HCC1937 cannot mediate double strand break repair [Zhang *et al,* 2004]. In contrast to *wild-type* PML, the mutant PMLSer117A does not sensitize U937 leukaemia cells to gamma irradiation- induced apoptosis suggesting functional importance for phosphorylation of Ser117 by Chk2. For both proteins, a mechanistic understanding of how modification impacts function is lacking. For example, it will be of interest to determine if the alanine mutants of either protein are able to inhibit downstream functions of Chk2.

Lukas and colleagues [2003] demonstrated using both fluoresence-loss-in-photobleaching (FLIP) and fluoresence-return-after-photobleaching (FRAP) that unlike other another checkpoint protein Nbs1, Chk2 does not become immobilized at sites of damaged DNA but remains soluble after x-irradiation. Further, immobilization of Chk2 by fusion to Histone2B inhibits Chk2 function leading to the view that Chk2 acts as a “signal spreader” dispersing a checkpoint “on” signal to its various substrates implementing cell cycle arrest and apoptosis [Lukas *et al*, 2003]. Given that both PML and BRCA1 form foci which collect many checkpoint proteins including Chk2’s principle activator

ATM [Xu *et al*, 2003; Wang *et al*, 2000], perhaps PML and BRCA1 function in part to provide sites of Chk2 activation by ATM. Moreover, Thr68-Chk2 colocalizes with 53BP1, H2AX, and NBS1 in nuclear foci [Wang *et al*, 2002; Ward *et al*, 2001]. Once activated therein, Chk2 disengages from the complex by phosphorylation of PML, BRCA1, or other factors to then find and activate Chk2's various substrates. Consistent with this model, BRCA1 is required for Thr68 phosphorylation by ATM [Foray *et al*, 2003]. A study, which found that closely associated mismatch repair factors (MMR) MSH2 and MLH1, members of the BASC or BRCA1-associated genome surveillance complex [Wang *et al*, 2000], bind Chk2 and ATM respectively and play an important role in Chk2 activation generally supports this hypothesis. Restoration of either MSH2 or MLH1 in colon tumor cell lines bearing mutations in these factors enhances Thr68 phosphorylation after DNA damage, restores Cdc25A destabilization by gamma irradiation, and complements the RDS phenotype, all hallmarks of increased Chk2 signaling [Brown *et al*, 2003]. Also, Chk2 from MSH-/- cells does not undergo Thr68 phosphorylation after gamma irradiation [Franchitto *et al*, 2003]. Moreover, Chk2 activation is defective in cells derived from Nijmegen Breakage Syndrome patients who carry inactivating mutations in Nbs1 a component of the Mre11–Rad50–Nbs1 complex which plays central role in DNA repair and is required for ATM activation after DNA damage [Buscemi *et al*, 2001; Usui *et al*, 2001; Uziel *et al*, 2003; Carson et al, 2003; Lee *et al*, 2003; Mochan et al, 2003] 53BP1 another checkpoint factor important in forming DNA damage signaling complexes with ATM and BRCA1 may also play a role in Chk2 activation. 53BP1 null MEFs show little if any defect in Chk2 phosphorylation

as determined by mobility shift in polyacrylamide gels after IR [Ward *et al*, 2003; Fernandez-Capetillo *et al*, 2002] but focus formation of activated Chk2 as detected by an anti-phosphoT68 Chk2 antibody is compromised by 53BP1 knockdown [Wang *et al*, 2002]. Interestingly, 53BP1 is involved in the phosphorylation of other ATM substrates including Smc1 [DiTullio *et al*, 2002]. Also, Chk2 activation is independent of H2AX which is phosphorylated at sites of DNA damage [Fernandez-Capetillo *et al*, 2002]. A more recently discovered checkpoint factor also present in damage foci, NFBD1/MDC1 binds to phospho-Chk2 but its requirement for Chk2 Thr68 phosphorylation is controversial [Lou *et al*, 2003; Stewart *et al*, 2003; Goldberg *et al*, 2003; Peng & Chen, 2003; Shang *et a.*, 2003]. Detailed understanding of how the various signaling interactions required for Chk2 activation are integrated will be of great interest.

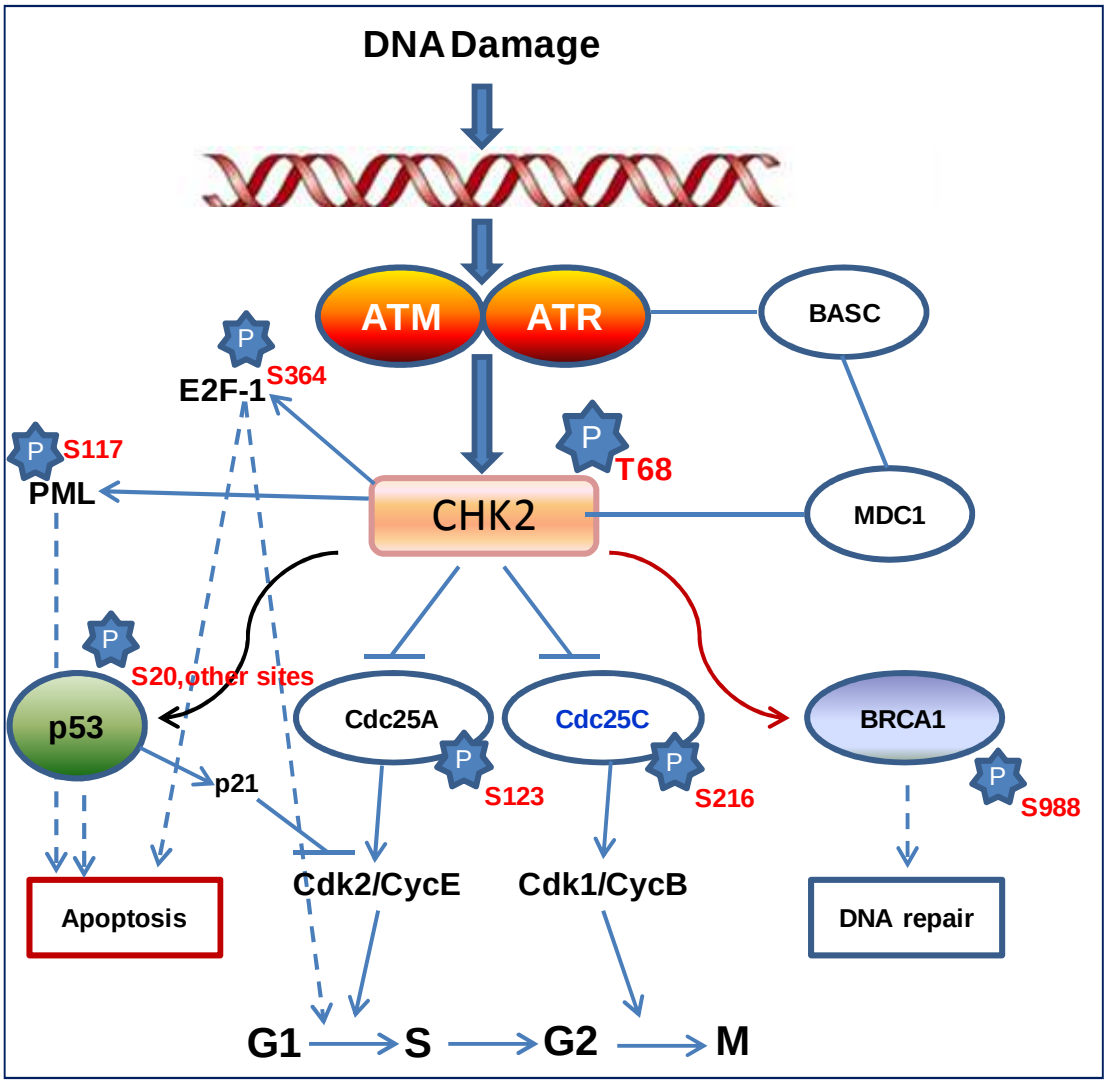

Figure 16. The Chk2 network.

Chk2 integrates signals from upstream kinases and binding partners to phosphorylate key targets involved in cell cycle checkpoints.

6.5. Chk2 knockout mice and p53 connection

Given the central role of both p53 and Chk2 in cell cycle arrest and apoptosis as well as their roles in tumor suppression, many reports have described connections between Chk2 and p53. Conclusions from several studies, however, have led to the realization that different approaches can provide widely varying results. Several groups placed Chk2 immediately upstream of p53 through studies involving Chk2 knockout mice as well as in vivo and in vitro experiments. Phosphorylation of p53 at Ser20 had been suggested to be a critical event for stabilizing p53 [Shieh *et al*, 1999; Unger *et al*, 1999]. The search for a kinase capable of Ser20 phosphorylation seemed to end when it was reported that Chk2 could phosphorylate p53 at Ser20 in vitro and this event was sufficient to disrupt pre-complexed p53 and Mdm2 [Shieh *et al,* 2000; Chehab *et al*, 2000]. Introduction of a dominant negative Chk2 led to failure of U2OS cells to stabilize or phosphorylate endogenous p53 at Ser20 and Chk2 enhanced a p53 dependent G1 checkpoint [Chehab *et al*, 2000]. In another study, it was reported that immunoprecipitated Chk2 is activated by IR to phosphorylate p53 and overexpression of Chk2 enhances the ability of p53 to transactivate a p53 responsive reporter plasmid [Falck et *al*, 2001]. Interestingly, Chk2 downregulation by antisense in 293 cells increases sensitivity to DNA double strand breaks [Yu *et al*, 2001]. Silencing of Chk2 by novel engineered zinc finger transcription factors decreases p53 transcriptional activation of the MDM2 gene and reduces p53 Ser20 phosphorylation [Tan *et al*, 2003].

However, other work has weakened the view that Chk2's regulation of p53 is generally applicable. First, the region on p53 spanning Ser20 does not share homology with the consensus motif, L-X-R-X-X-S/T present in other known substrates of Chk2 and a peptide covering the p53 Ser20 region is a poor Chk2 substrate [O'Neill *et al,* 2002; Seo *et al*, 2003]. Interestingly, two regions of p53, aa 117–132 and aa 272–288, are proposed to provide Chk2 docking sites to allosterically regulate the kinase activity toward p53 and purified Chk2 supplemented with these docking-site motifs phosphorylated a Ser20 peptide by kinase assay [Craig *et al*, 2003]. A second group has identified another Chk2 binding domain in p53 centering on Thr329 [Qin *et al*, 2003]. In contrast to this work, Chk2 purified from tumor cell lines before and after DNA damage could not phosphorylate different forms of truncated or full-length p53 even though it was markedly activated to phosphorylate the domain of Cdc25C containing Ser216 [Ahn *et al*, 2003]. Experiments in vivo similarly challenge the Chk2–p53 relationship. Introduction of Chk1 and Chk2 siRNAs into several human tumor cell lines failed to affect p53 stabilization, Ser20 phosphorylation or transcriptional activity despite preventing Ser216 phosphorylation of Cdc25C [Ahn *et al*, 2003]. Even more, homozygous deletion of Chk2 in HCT-116 colon carcinoma cells yielded no phenotype with respect to p53, G1 or G2 cell cycle arrest, and apoptosis [Jallepalli *et al*, 2003].

Two independently generated Chk2 knockout mice have supported a role for Chk2 in the regulation of p53 but diverge in their findings and proposed mechanisms. The first reported Chk2 null mouse from Hirao and colleagues [Hirao *et al*, 2000] strikingly showed that thymocytes, splenic T cells, and

MEFs lacking Chk2 are unable to stabilize p53 following gamma irradiation and this defect is corrected by reintroduction of Chk2. These cells are highly resistant to apoptosis consistent with a defect in p53 signaling [Hirao *et al*, 2000]. A follow up study showed that Chk2 mice are highly resistant to apoptosis in multiple tissue compartments by whole body irradiation experiments and develop chemically-induced skin tumors more rapidly [Hirao *et al*, 2002]. The G1/S checkpoint of these mice is compromised at low doses of gamma irradiation but cells arrest normally at doses above 5Gy. Interestingly, however, p53 null mice do not show any dose dependency in the same assay. In contrast to the original report a second study from this group showed that gamma irradiated Chk2-/- thymoctyes show a defect in transcriptional activation of Bax but only a slight depression in p21 upregulation [Hirao *et al*, 2002].

A different group using MEFs from these same mice, however, found no defect in G1/S arrest or p21 mRNA induction at even low X-ray doses [Jack *et al*, 2002]. More recently a second Chk2 knockout mouse generated by another group was reported with somewhat different results [Takai *et al*, 2002]. Consistent with the first Chk2 null mouse, the second was resistant to apoptosis induced by gamma irradiation in many tissues strongly implicating Chk2 in DNA damage induced apoptosis [Takai *et al*, 2002].

No G2 or S-phase checkpoint defect was detected but these authors did observe a deficient G1/S arrest consistent with impaired p53 signaling. In contrast to Hirao et al, they reported only a 30–50% reduction in p53 stabilization and human p53 introduced into Chk2-/- MEFs was phosphorylated normally on Ser20 after IR, indicating that in murine tissue

Chk2 makes a partial contribution to p53 stabilization but other factors clearly contribute. Perhaps the most striking result with respect to p53 was that, despite only a partial p53 stabilization defect, upon analysis of five p53 targets genes by quantitative RT–PCR in thymocytes and MEFs, the authors saw no mRNA upregulation in the Chk2-/- cells after IR providing a possible mechanistic explanation for the G1/S arrest and apoptotic defects in these cells [Takai *et al*, 2002]. However, the relationship between Chk2 and p53 is likely to be stimuli–as well as cell type-specific as cell death in primary neuronal cultures induced by etoposide or camptothecin was shown to be ATM-dependent but Chk2 independent [Keramaris *et al*, 2003].

To summarize the mouse studies to date, it is clear that Chk2 plays a central role in gamma irradiation induced apoptosis however the mechanism is unclear. Chk2 likely regulates p53 transcriptional activity independently of Ser20 phosphorylation or even stabilization in murine tissues after IR, but in other contexts yet to be described signaling pathways play a central role. It remains to be determined when Chk2 regulates p53, in what context, and what factors mitigate or compensate for Chk2 in cases where it is not required.

6.6. Targeting DNA damage checkpoint kinases as anticancer treatment

Radiation therapy and chemotherapy have been used as important modalities for anti cancer treatment for decades. It is likely that they will remain in use for anti cancer therapy in the foreseeable future. Their central function is to damage DNA. The damaged DNA will immediately activate the DDR network, and thus hamper the desired effects, while cancer growth arrest and apoptosis are major outcomes desired in drug treatment. Since damage to DNA might be the common underlying mechanism for the positive outcome of anticancer therapy, an important issue emerging in drug discovery is to target anticancer treatments on cell cycle checkpoints. Recently, we could show that besides the epigenetic regulation of the $p21^{WAF1}$ promoter [Habold *et al,* 2008], G_2 checkpoint arrest is not only induced trough the checkpoint kinase Chk1, but early Chk1 activation also directs senescence and mitotic catastrophe – two main strategies to long-term affect the growth of tumor cells – even in recovery from G_2 checkpoint arrest [Poehlmann *et al*, 2011]. Furthermore, inhibition of important molecules in the DNA damage response network has increasingly been considered as an innovative strategy for improving the effects of chemotherapy and radiation therapy [Janetka *et al*, 2007; Bulavin *et al*, 2001; Tao & Lin, 2006]. Significant experience in this field was already gained in the 1990s. At that point of time, it could be shown that caffeine is an inhibitor of ATM and ATR [Sarkaria *et al*, 1999]. However, the high dosage of caffeine required for this function cannot be applied to humans.

Chk1 with its central function in DNA damage response pathways has also been considered as an appropriate target for inhibition strategies. In the last several years, a large number of chemical inhibitors to Chk1 have been developed [Janetka et al, 2007]. These substances inhibit, in addition to Chk1, a variety of kinases, including Chk2. The therapeutic possibilities of targeting Chk1 are discussed in a recent review [Janetka et al, 2007]. The possibilities and also problems caused by applying Chk2 inhibitors as therapeutic approach in clinical oncology have recently been reviewed by Antoni *et al*. [2007]. To optimize the effects of chemotherapy and radiation therapy, inhibitors to a larger number of other checkpoint kinases are also being developed.

In addition, because of its importance in cancer, the ERK pathway has been a focus for drug discovery, but rather with Ras, Raf, and MEK as the main targets [Downward, 2003] and [Kohno & Pouyssegur, 2006]. Furthermore, inhibition of p38 activity causes enhanced apoptosis in response to DNA-damaging agents, such as doxorubicin and cisplatin, as well as microtubule-disrupting agents, such as taxol, vicristine, and vinblastine [Deacon *et al*, 2003; Lee *et al*, 2006; Losa *et al*, 2003]. In this context, also JNK inhibitors have been considered for anticancer therapy, and this is linked to their ability to interfere with DNA repair in response to genotoxic drugs [Kennedy *et al*, 2003]. However, as JNK may also have a tumor suppressor function due to its ability to promote apoptosis, JNK inhibitors would then prevent apoptosis, and thus the usefulness of JNK inhibitors is still under discussion.

6.7. Exploiting CHEK2 genetics in cancer treatment

A key issue now is whether our current knowledge about CHEK2 and tumour susceptibility can be used to help treat patients with cancer. Testing most cancer patients for mutations in CHEK2 is probably not yet appropriate, as the evidence indicates that CHEK2 on its own does not predispose to cancer. However, if it is correct that CHEK2 mutations are associated with an increased risk of contralateral breast cancer and with breast cancer developing from benign tumours after radiotherapy, knowing the CHEK2 status of such patients might help in their management. Finally, is it possible to exploit the loss of CHK2 function as a therapeutic strategy? One possibility may be the use of poly(ADP-ribose) polymerase (PARP) inhibitors in cancer patients who carry somatic but not germline mutations in CHEK2, as it has been reported that tumour cells that are functionally deficient for CHK2 are sensitive to PARP inhibition109. Indeed, PARP inhibitors are currently being evaluated as a cancer therapy in those individuals who are deficient for BR CA1 and BRCA2, which function in the same repair pathway as CHK2.

6.8. Drugs targeting CHK2: inhibition or activation?

There is currently a debate over the most appropriate way to target CHK2 in tumours that harbour the *wild type* form of this kinase. Among the issues are questions of compound selectivity and the potential for combination with

DNA-damaging chemotherapy and radiotherapy. At the cellular level it is necessary to take account of the different molecular lesions caused by different DNA-damaging agents, which elicit distinct responses from the CHK2 pathway. The context of intervention is also important, particularly the tumour genetic background such as p53 mutational status. The level of intrinsic DNA damage in a given tumour cell type and the degree to which CHK2 functions might be essential for maintenance of the transformed phenotype have to be considered. In circumstances of high intrinsic DNA damage, a CHK2 inhibitor might have the potential for single-agent efficacy. However, in tumours where activated CHK2 contributes directly to the malignant phenotype or to resistance to DNA-damaging agents, a combination of a CHK2 inhibitor with a DNA-damaging agent might lead to an increased therapeutic index. There are also suggestions that hyperactivation of CHK2 pathways, in the absence of extrinsic DNA-damaging agents, would be effective for inducing certain tumour cells into senescence or even apoptosis.

6.8.1. Inhibition of CHK2

There are several lines of evidence that suggest that CHK2 inhibition in combination with genotoxic agents (IR and chemotherapeutics) might have therapeutic value. Inhibition of CHK2 expression has been found to attenuate DNA damage-induced cellcycle checkpoints and to enhance apoptotic activity in HEK293 cells [Rose *et al*, 2001]. CHK2 inhibition has also shown

activity in two cellular models of mitotic catastrophe, indicating a potential for chemosensitization with doxorubicin [Castedo *et al,* 2004].

In a recent report, targeting of CHK2 using small interfering RNA or a dominant-negative form of this kinase (CHK2-DN) prevented survivin release from the mitochondria and enhanced apoptosis following DNA-damage by IR or doxorubicin [Ghosh *et al*, 2006]. In addition, the conditional expression of CHK2-DN showed potentiation of doxorubicin cytotoxicity in the HCT116 colon carcinoma cell line grown as a xenograft in mice. It should not be overlooked, however, that several studies have reported no therapeutic value of CHK2 inhibition. In particular, it has been reported that loss of CHK2 function has no additional therapeutic benefit compared with loss of CHK1 alone when combined with the antimetabolites 5-fluoro-2-deoxyuridine and gemcitabine [Morgan *et al*, 2006]. Similarly, small interfering RNA studies in two cancer cell lines have led to the proposal that CHK1 is the only checkpoint kinase that is relevant as a drug target [Xiao *et al,* 2006]. In part, this underscores the difficulty of reconciling results from multiple cell line models, using non-equivalent DNAdamaging agents and different strategies for inhibition of CHK2 function. It may be that complete validation of this target in the context of cell killing in vitro and in vivo must await pharmacological studies with selective smallmolecule inhibitors of CHK2 in tumours of defined genetic background.

To date, all reported inhibitors of CHK2 target the ATP-binding pocket of this kinase. This is not surprising as most compounds have been identified through screening for inhibitors of the catalytic activity of this protein. As a result, CHK2 inhibitors tend to inhibit other kinases in addition to their primary

target, and in particular the functional relative, CHK1. This has meant that the difficulty of examining the CHK2- dependent pathways separately from CHK1-mediated responses has been a recurrent theme in the pharmacological validation of CHK2 as a drug target. For example, the dual CHK1 and CHK2 inhibitors Go6976 and EXEL-9844 (currently in clinical trials) showed effects in cellular assays that are also seen with loss of CHK1 alone and so it is not possible to ascertain from these studies the independent effect of CHK2 inhibition [Kohn *et al*, 2003; Matthews *et al*, 2007].

The advent of selective inhibitors of CHK2 promises to address this problem and the recent publication of the X-ray crystal structure of the CHK2 kinase domain will be useful in the development of additional chemical classes of CHK2-selective inhibitors [Oliver *et al*, 2006]. A recent report identified a series of bis-guanylhydrazone compounds as potent CHK2 inhibitors using in vitro kinase assays [Jobson *et al*, 2007], although these inhibitors did not show inhibition of CHK2 in cells and might suffer from confounding off-target activities or poor cell permeability. In addition, a series of isothiazole carboxamides has provided selective (versus CHK1) CHK2 inhibitors, such as VR X0466617 [Larson *et al*, 2007; Carlessi *et al*, 2007]. This small molecule clearly blocked CHK2 activity in cells, but did not significantly change the cell-cycle distribution or prevent the G2/M arrest in short-term culture of normal or irradiated cells. In longer-term culture (>6 days) exposure of normal cells to VR X0466617 alone led to an antiproliferative effect, but the possibility that this was a manifestation of an off-target activity could not be ruled out. Evaluation of this compound in

combination with doxorubicin or cisplatin showed no potentiation of cytotoxicity. It should be noted, however, that the combination studies were performed in the MCF-7 cell line, which harbours wild-type p53. It will therefore be interesting to test whether selective and potent inhibitors of CHK2 potentiate cytotoxic treatments in p53-deficient cell lines.

One therapeutic strategy in which inhibition of CHK2 has clear value is radioprotection. As discussed earlier, targeted disruption of Chk2 in irradiated mice leads to increased survival through the suppression of apoptosis [Takai et al, 2002; Hirao *et al*, 2000; Hirao *et al.*, 2002; Jack *et al*, 2002]. This led to the hypothesis that targeting CHK2 with a small-molecule inhibitor might suppress the side effects of radiotherapy, which is administered to approximately 50% of all cancer patients. The first pharmacological validation of this therapeutic strategy came with a report on a series of selective ATP-competitive 2-arylbenzimidazole CHK2 kinase inhibitors, which were developed as radioprotective agents for normal proliferating tissues [Arienti *et al*, 2005]. A potent 2-arylbenzimidazole prevented γ-irradiation-induced apoptosis of CD4+ and CD8+ human T cells isolated from blood at doses consistent with the biochemical measurement of CHK2 inhibition. This radioprotective effect has also been reported with the structurally distinct VR X0466617 CHK2 inhibitor in isolated mouse thymocytes [Carlessi *et al*, 2007]. The observation of radioprotection of normal cells with two distinct and selective CHK2 inhibitors suggests that this is a promising therapeutic context to pursue.

6.8.2. Activation of CHK2

As an alternative to CHK2 inhibition, there may also be circumstances where activation of this kinase could have therapeutic value. CHK2 clearly has a role in the barrier to oncogenesis and its activation in the absence of DNA-damaging agents may force tumour cells to exit the proliferative state, either through death or senescence. In a study investigating this experimental therapeutic approach [Chen *et al*, 2005], involved stable transfection of p53-deficient DLD1 colon cancer and HeLa cervical carcinoma cell lines with a tetracycline-inducible form of CHK2. On induction, CHK2 was produced in both cell lines and activated owing to phosphorylation at T68 in the absence of DNA-damaging agents, perhaps due to a background level of constitutive DNA damage or through oligomerization of the protein [Oliver et al, 2006]. The increased levels of activated CHK2 led to decreased cell proliferation, G2 arrest and increased apoptosis.

Additionally, by eye the cells looked senescent. Thus, manipulation of CHK2 activation, for example, through inhibition of the PPM1D/WIP1 phosphatase (which antagonizes CHK2 activation through T68 dephosphorylation) might have therapeutic benefit. However, a note of caution is needed as this study involved overexpression of CHK2 and so it may be that only the introduction of large quantities of CHK2 into cells and not chemical activation of the endogenous kinase will allow senescence or death. Altogether, cellular and pharmacological studies demonstrate that the response of a tumour to CHK2 manipulation will depend on a specific cellular context. Given that many tumour cells exhibit high levels of intrinsic DNA

damage, the functional availability of the DNA-damage response and repair components will dictate the therapeutic outcomes of CHK2 inhibition or activation. This is exemplified by a recent study showing that pharmacological inhibition of the repair machinery in combination with IR results in increased activation of CHK1 and CHK2 [Sturgeon *et al*, 2006]. Consequently, the ability of checkpoint inhibitors to abrogate the G2 arrest and sensitize these cells to IR was reduced compared with their activity in irradiated cells not exposed to inhibitors of DNA repair. These results highlight the importance of considering the balance of all components in the CHK2 pathway: the levels of DNA damage, the degree of signalling through CHK2 and the efficiency of DNA repair in response to checkpoint activation.

Chapter 2

AIM OF THE STUDY

Survivin is a pivotal cancer gene with multiple roles in cell viability and mitosis, but the function(s) of its alternatively spliced isoforms has remained elusive.

Transcription of the *survivin* locus is complex, giving rise to at least five mRNA species that encode, in addition to *wild type* (WT) survivin, the variants survivin-2B, -3β, -2α and -ΔEx-3. Structurally, survivin-2α and -3B are generated by "read-through" into intron 2, or via inclusion of an alternative exon 3B, whereas survivin-2B and -ΔEx3 originate from the insertion of an alternative exon 2B , or the skipping of exon 3, respectively.

Elucidating the function(s) of the survivin spliced variants has not been straightforward, complicated by the low levels of expression of these molecules in most cells, and the limited availability of specific reagents that discriminate between the different isoforms. For instance, survivin-2B has been reported to promote apoptosis, *in vitro*. However, low levels of survivin-2B correlate with better survival in acute myeloid leukemia, and its silencing in ovarian cancer has been linked to enhanced sensitivity to taxanes in resistant tumors. A role of the survivin isoforms in mitosis has been equally controversial, as this function has been proposed in some reports, but negated in others.

In this study, we took a multidisciplinary approach of genome-wide bioinformatics, analysis of the DNA damage response and evaluation of primary patient samples, to dissect a potential role of survivin-ΔEx3 in cancer.

Chapter 3

MATERIAL & METHODS

1. Drugs

Cycloheximide, camptothecin and etoposide were purchased from Sigma (Saint Louis, Missouri, USA).

1.1. Cycloheximide Preparation

To obtain a 2 mg/ml cycloheximide stock solution, 10 ml of sterile water were added to 20 mg cycloheximide powdered stock and the drug was completely dissolved, stored at -20℃ and diluted i n complete culture medium immediately before use.

1.2. Camptothecin Preparation

To obtain a 5 mg/ml camptothecin stock solution, 10 ml of DMSO were added to 50 mg camptothecin powdered stock and the drug was completely dissolved, stored at -20℃ and diluted in complete culture medium immediately before use.

1.3. Etoposide Preparation

To obtain a 5 mg/ml etoposide stock solution, 10 ml of PBS were added to 50 mg etoposide powdered stock and the drug was completely dissolved, stored at -20℃ and diluted in complete culture med ium immediately before use.

2. Human Tumour Cell Lines

The human lung adenocarcinoma (H460), breast adenocarcinoma (MDA-MB-431 and MCF-7), glioblastoma (LN229), and colorectal adenocarcinoma (HCT116 and SW480) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA).

The colorectal adenocarcinoma cell line HCT116 containing the DR-GFP construct was kindly provided by Dr. S. Powell (Memorial Sloan Kettering Center, New York, NY).

2.1. Human Tumour Cell Lines Growth Conditions

All cell lines were maintained as monolayers in 100x20mm Petri Dish (Corning, Pittsburgh, PA, USA) at 37℃ in a 5% CO_2 humidified atmosphere in an air incubator (Thermo Fisher Scientific, Pittsburgh, PA, USA).

H460 were maintained in RPMI-1640 medium (Invitrogen, Grand Island, NY, USA), supplemented with 10% (v/v) FBS. LN229, MDA-MB-431 and MCF-7 cells were cultured using D-MEM medium (Invitrogen), supplemented with 5% (v/v) FBS. HCT116 were maintained in Mc Coy's 5a medium (Invitrogen), supplemented with 10% (v/v) FBS and SW480 were cultered using Leibovitz's L-15 medium supplemented with 10% (v/v) FBS.

All tumour cells were cultured as follow: at set time points, specific growth medium was removed from the 100x20mm Petri Dish and the cell monolayer was washed twice with 5 ml 37℃-heated PBS. One ml of a Trypsin-EDTA solution (Invitrogen) was then added to each dish. After a 5 minutes-incubation at 37℃ with gentle shaking, cell detach ment was checked under the microscope (Micromaster, Fisher Scientific, Pittsburgh, PA, USA) and trypsin activity was blocked by addition of 5 ml of complete medium. Five ml-suspension containing detached cells was transferred in a sterile 15 ml conical tube (Falcon-Fisher Scientific) and centrifuged at 1,500 rpm for 5 minutes at room temperature in a Beckman GS-6R refrigerated centrifuge (Beckman, Danvers, MA, USA). Cell pellet was then carefully resuspended in 5 ml of growth medium and a small aliquot (10 µl) was checked under the microscope in a Burker camera, and the number of detached cells was calculated by counting the number of cells after Trypan blue coloration.

Frozen stocks of human tumour cell lines were prepared as follows. Cells in logarithmic growth phase were detached from the dishes and counted as previously described. The percentage of viable cells was also determined by Trypan blue dye exclusion test by mixing, in a 0.5 ml clean conical tube (Eppendorf – Fisher Scientific) 5 µl of detached cells suspension, 45 µl of

PBS and 50 µl of Trypan blue dye (Invitrogen). Five µl of Trypan blue dye-stained cell suspension were placed onto a Burker camera and checked under the microscope. The number of viable cells generally exceeded 95%. After counting, the cell suspension was centrifuged at 1,500 rpm for 5 minutes at 4℃. The supernatant was removed and the cell pellet was carefully resuspended in 4℃-cold freezing medium [D-MEM/F12 medium supplemented with 40% (v/v) FBS] at a density of 5 x 10^6 viable cells/ml. One ml of such a cell suspension was then transferred in a 2 ml sterile Nalgene tube (Nalgene Company, Rochester, NY, USA) and placed on ice. One ml of cryoprotective medium [basal EAGLE'S medium with HANKS' BBSS and 15% (v/v) dimethylsulfoxide (DMSO)] (Invitrogen) was added to each tube and after a gentle resuspension, the tube was placed onto a Cryo 1℃ Freezing Container (Nalgene) and stored at –80℃ for the initial freezing step. After 16-24 hours, frozen cells were removed from the freezing container and stored under liquid nitrogen vapours in the Barnstead/Thermolyne containers (Barnstead/Thermolyne, Dubuque, IO, USA).

For cell thawing, frozen cells were incubated in a 37℃ water bath (Thermo Fisher Scientific), transferred into a sterile 15 ml conical tube containing 10 ml of complete growth medium and centrifuged at 1,500 rpm for 5 minutes at room temperature. The cell pellet was then resuspended in 15 ml of growth medium and cells were seeded in a 100x20mm Petri Dish.

3. Nucleic Acids Quantification

Concentration and quality of solutions containing DNA, RNA or oligonucleotides were determined by spectrophotometric analysis as follows. In two clean 1.5 ml tube (Eppendorf), a suitable volume of nucleic acid-containing solution (generally, 5 and 10 µl) was gently mixed with RNase- or DNase-free distilled water to a final volume of 200 µl to obtain a 1:40 and 1:20 dilutions. As the blank sample, a third clean tube containing 200 µl of RNase- or DNase-free distilled water was also prepared. The content of each tube was transferred into quartz cuvettes (Fisher Scientific), the cuvettes were then placed onto a Lambda 11/Bio spectrophotometer (Fisher Scientific) and absorbance values at 260 nm (A_{260nm}) and 280 nm (A_{280nm}) of wave length were measured. The A_{260nm} and A_{280nm} values obtained with the blank sample were subtracted from the absorbance values obtained with the nucleic acid-containing samples, and DNA, RNA or oligonucleotide concentration was then calculated using these formulae:

µg/ml of DNA = (A_{260nm}) x (DILUTION FACTOR) x (50 µg/ml)

µg/ml of RNA = (A_{260nm}) x (DILUTION FACTOR) x (40 µg/ml)

µg/ml of oligonucleotide = (A_{260nm}) x (DILUTION FACTOR) x (30 µg/ml)

The nucleic acid concentration was expressed as the mean of the values (µg/ml) obtained in the two independently-prepared and –measured dilutions.

The quality of nucleic acid solutions was evaluated by calculating the A_{260nm}/A_{280nm} ratio. It is well known that for both DNA and oligonucleotide, a

ratio of 1.8-2 was indicative of a good-quality preparation, whereas high quality RNA solutions are characterised by a A_{260nm}/A_{280nm} ratio of 2.

4. Protein Concentration Determination

Protein concentration was determined according to Bradford [1976].

4.1. Calibration Curve Preparation

Solutions of bovine serum albumin (BSA) (0.001 µg/µl, 0.0025 µg/µl, 0.005 µg/µl, 0.01 µg/µl, 0.025 µg/µl and 0.05 µg/µl) were prepared by dissolving powdered BSA (Sigma) in distilled water. In a 1.5 ml tube, 250 µl of each BSA solution were mixed with 150 µl of distilled water and 100 µl of Biorad Protein assay dye (Biorad, Hercules,CA, USA). In the blank sample, 400 µl of distilled water were mixed with 100 µl of Biorad Protein assay dye. Samples were rapidly transferred into disposable 96 well plate (Corning-Costar) and the absorbance at 595 nm wave length of each calibration point was measured using a microtiter plate reader (Fisher Scientific). The absorbance value corresponding the blank sample was then subtracted from the values obtained in the BSA-containing samples. Each calibration sample was run in triplicate.

4.2. Proteins Concentration Quantification

Concentration of extracted proteins was determined by mixing in a 1.5 ml tube 5 µl of protein extracts with 95 µl of Biorad Protein assay dye and distilled water to a final volume of 500 µl. Samples were rapidly transferred into 96 well plate and the absorbance at 595 nm wave length was measured in the spectrophotometer plate reader. From the calibration curve, it was possible to determine the protein concentration of each sample. Also in this case, each sample was run in triplicate.

5. Agarose Gel Preparation

All agarose gel electrophoresis experiments were carried out using Hoefer horizontal electrophoresis units (Biorad).

Gels of different sizes and percentages (w/v = grams of powdered agarose per 100 ml of running buffer) were prepared in clean glass bottles by mixing suitable amounts of powdered agarose (Sigma) in 1x TAE [0.04 M Tris-Acetate (Sigma), 0.001 M EDTA (Sigma)] or 1x TBE [0.09 M Tris-Borate (Sigma), 0.002 M EDTA] running buffers. Agarose was dissolved by heating in a microwave, cooled to about 50℃, transferred o nto the casting tray and, after the insertion of a clean comb, left to polymerise at room temperature. When polymerisation was completed, the comb was removed and solidified

agarose gel was transferred into the running tray and submerged with running buffer.

6. SDS-Polyacrylamide Gel Preparation

All SDS-polyacrylamide gels used for protein separation (8 centimetres high/ 10 centimetres length/ 1 millimetres thick) were prepared as described by Laemmli [1970] and run in a vertical electrophoresis slab unit (Biorad). Resolving gel (10 ml) was prepared in a 15 ml conical tube (Falcon-Fisher Scientific) by mixing a suitable volume of 40% (w/v) polyacrylamide stock solution (acrylamide:N,N'-methylenebisacrylamide molar ratio = 29:1) (Sigma) with 0.1 ml of 10% (w/v) SDS solution (Sigma) [final concentration = 0.1% (w/v)], 2.5 ml of 1.5 M Tris-HCl pH 8.8 solution (Sigma) (final concentration = 375 mM), 100 µl of 10% (w/v) APS (Sigma) [final concentration = 0.1% (w/v)] and 6 µl of TEMED (Sigma) [final concentration = 0.06% (v/v)], and subsequently poured into the gap between glass plates (previously washed with absolute ethanol) leaving sufficient space for the stacking gel. The poured resolving polyacrylamide gel solution was immediately and carefully overlaid with 0.1% (w/v) SDS solution (the overlay prevents oxygen from diffusing into the gel and inhibiting polymerisation) and the gel was placed in a vertical position at room temperature to polymerise. When polymerisation was complete (within 20-40 minutes), the overlay was poured off and the top of the gel was carefully washed several times with

distilled water to remove any unpolymerised acrylamide. The stacking gel (2 ml) containing 5% (w/v) polyacrylamide (acrylamide:N,N'-methylenebisacrylamide molar ratio = 29:1), 0.1% (w/v) SDS, 125 mM Tris-HCl pH 6.8, 0.1% (w/v) APS and 0.1% (v/v) TEMED was prepared in a 15 ml conical tube and poured directly onto the surface of the polymerised resolving gel. A clean comb was immediately inserted into the stacking gel solution and the gel was placed in vertical position to polymerise. When polymerisation was complete, the comb was removed, the wells were washed with distilled water to remove any unpolymerised acrylamide and the gel was mounted in the electrophoresis apparatus containing TGS running buffer [25 mM Tris base, 250 mM Glycine (Sigma), 0.1% (w/v) SDS, pH 8.3] in both upper and lower buffer reservoirs.

7. Preparation of the Plasmid Vectors

To obtain a permanent and stable source of pcDNA3 (Invitrogen, San Diego, CA, USA), pGEX4T (Invitrogen), survivin-cDNA, survivin-ΔEx3 cDNA vectors, the following procedures were performed.

7.1. Transformation of Bacteria

In an ice-cold 1.5 ml Eppendorf tube, 50 µl of competent bacterial cells (DH5α, Invitrogen) were mixed with 50 ng (5 µl). The mixture was gently mixed by tapping and the tube was chilled on ice for 30 minutes, incubated for 20 seconds at 42℃ in a water bath and for 2 minutes on ice. After a 5-minute incubation at room temperature, 900 µl of LB medium were added and tube was placed into a 37℃-heated shaking incubator at 225 rpm for 2 hours. The tube was then centrifuged at 3,000 rpm for 5 minutes at room temperature, 950 µl of supernatant LB were removed and bacterial cell pellet was resuspended in the remaining 50 µl, plated onto 90-mm dish (Corning-Costar) containing selective solid LB medium [liquid LB medium, 1.5% (w/v) agar (Invitrogen), 50 µg/ml ampicillin (Sigma)] and incubated over night at 37℃. Each colony, representing ampicillin-resistant/transformed growing bacterial cells, was picked-up with a sterile disposable loop, dissolved in a 50 ml conical tube containing 10 ml of liquid LB medium supplemented with ampicillin (50 µg/ml final concentration) and allowed to grow over night at 37℃ with 225 rpm shaking.

7.2. Small Scale Preparation of Plasmid DNA (“miniprep”)

To confirm the presence of plasmids in ampicillin-resistant liquid culture, 8 ml of the bacterial suspension were used for “miniprep” by using Qiagen

Plasmid Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Plasmid DNA concentration was determined spectrophotometrically as described in the section 1.3.

Electrophoretic analysis was performed on 1% (w/v) agarose mini gel prepared and run in 1x TAE buffer. About 500 ng of extracted and original DNA vectors were loaded and separated at 90 volt (SE300 power supply; Amersham) at room temperature for 1 hour in the presence of 500 ng of *λ-BsteII* molecular weight marker (Sigma). DNA fragments were visualised by placing the gel onto the UV-transilluminator (260 nm wave length) (Biorad) after a 30-minutes staining with 1 µg/ml ethidium bromide (Invitrogen)-1x TAE buffer staining and a 30-minute 1x TAE buffer destaining.

7.3. Storage of Vector Transformed Bacteria

In a 2 ml sterile Nalgene tube, 1.6 ml of ampicillin-resistant liquid bacterial culture and 0.4 ml of sterile pure glycerol (final concentration (v/v) = 20%) were mixed by a vortex mixer and frozen by snap freezing in liquid nitrogen and stored at -80℃.

7.4. Large Scale Preparation of Plasmid DNA ("maxiprep")

By means of a sterile spatula, frozen bacterial cells were scraped from the 2 ml sterile Nalgene tube and dissolved in 10 ml of 50 µg/ml ampicillin containing-LB medium (contained in a 50 ml conical tube) and allowed to grow at 37℃ in a shaking incubator at 225 rpm. After 8 hours, 10 ml liquid culture was mixed with 100 ml of fresh 50 µg/ml ampicillin containing-LB medium into a 500 ml glass bottle and cells were left to grow over night at 37℃ and 225 rpm.

Bacterial cells were pelleted by a 30-minute centrifugation at 4,000 rpm at 4℃ and, after removal of LB medium, plasmid DNA was purified with the Qiagen Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions.

Concentration and quality of the plasmid DNA were evaluated as described in the section 3.

8. Bioinformatics analysis

We examined 14 cancer-related datasets with a total of 702 samples assayed on GPL5188 (Affymetrix Human Exon 1.0 ST) arrays for expression of survivin-ΔEx3 [Li,2005; Sampath & Pelus,2007]. Of the 14 datasets, 9 compared cancer or cancer-related cells with normal controls, and 5 compared either different cancers or the same cancer at different stages (Supplementary Table 1). The data were used as preprocessed and

normalized by the GEO contributors, and any technical replicates were averaged.

The HuEx-1_0-st Affymetrix microarray platform contains 22 probesets designed to detect sequences derived from the three isoforms of the *survivin* locus (Figure 1A): NM_001168 (survivin), NM_001012270 (survivin-ΔEx3) and NM_001012271 (survivin-2B). Of the 22 probesets, 9 were retained in all 14 datasets, and 13 were removed due to low expression (below background). Probesets 3 and 16 were also removed as their expression profiles were the same as 6 other probesets that targeted the same isoforms. Of the remaining probesets (Figure 1A), 8 of the 9 probes targeted regions that were common to all three survivin isoforms. Probeset 9 specifically targets exon 3, which is deleted in survivin-ΔEx3 [Li,2005; Sampath & Pelus,2007]. Specific expression of survivin-ΔEx3 was calculated as the difference between the average expression of the 8 common survivin probesets and probeset 9.

9. Antibodies

The following antibodies to checkpoint kinase 2(Chk2, Santa Cruz, Santa Cruz, CA, USA), Thr68 phosphorylated Chk2 (Cell Signaling, Danvers, MA, USA), survivin (Novus Biologicals, Littleton, CO, USA),p53 (Calbiochem, Rockland, MA, USA), p53 and Ser15-phosphorylated p53 (Cell Signaling), p21 (Calbiochem), Ser139-phosphorylated histone H2AX (γH2AX; clone

JBW301, Millipore, Billerica, MA, USA), Alexa Fluor® 488 (cat. # A-11017, Invitrogen, Carlsbad, CA), FLAG (Sigma-Aldrich), β-actin (Sigma-Aldrich), COX IV (Cell Signaling), and RCC1 (Santa Cruz) were used.

10. Mutagenesis

Mutagenesis of a 1.6 kb human survivin-ΔEx3 cDNA was carried out using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacture instructions. Ala substitutions of predicted Chk2 phosphorylation sites Thr79→Ala, Thr127→Ala, and Ser98→Ala in the unique –COOH terminus sequence of survivin-ΔEx3 was carried out with the following oligonucleotides (mutated sequences underlined):

5’-ATGCAAAGGAAACCAGCAATAAGAAGAAAGAAT-3’

(Thr79,ACA→GCA),

5’-TTATTCCCTGGTGCCGCCAGCCTTCCTGTGGGC-3’

(Thr127,ACC→GCC),

5’-AATCCATGGCAGCCAGGCGCTCGATGGCACGGC-3’

(Ser98,AGC→GCC).

Primers were purchased by IDT (Coralville, IA, USA). PCR products were then loaded on agarose gel (2%), the bands were excised and PCR products were purified with QIAquick® Gel Extraction Kit (QIAGEN) according to the manufacture’s instructions.

Mutant constructs were confirmed by DNA sequencing, and inserted into pcDNA 3.0 or pGEX4T vectors, using EcoRI and XhoI restriction sites.

11. Transfections

Twenty-four hours prior to transfection, cell types were seeded in 2 ml of fresh media at a density of 2 x 10^6 cells in 6-well plates (Corning-Costar). Just prior to transfection, the medium was removed and the fresh PBS was gently added in order to wash the cells. In a 0.5 ml tube, 4 µg of various FLAG-tagged (DYKDDDDKC) cDNA constructs and Lipofectamine2000™ (Lipo 2000), (Invitrogen) were incubated with 250 µl Opti- MEM® I (Invitrogen) per well at room temperature for 10 minutes. After the incubation, the diluted plasmids were combined with the diluted Lipo2000 (total volume is 500 µl) and incubated for 20 minutes. at room temperature to allow the plasmids:Lipo2000 complexes to form. This solution was then added to the cells and the plate was gently swirled to ensure uniform mixing. After 4 hours incubation at 37℃ in a 5% CO_2 humidified atmosphere, the transfection cocktail was washed out and replaced with fresh medium.

Gene silencing by small interfering RNA (siRNA) was carried out with control (VIII) or SMART pool siRNA oligonucleotides directed against Chk2 or survivin-ΔEx3 (Dharmacon), by oligofectamine (Invitrogen).

The day before transfections, each cell line was seeded at a density of 2 x 10^6 cells in 6-well plates (Corning-Costar). A given amount of each siRNA

was diluted in 200 µl of Opti-MEM® I reduced serum medium (Invitrogen) and incubated for 15 minutes. At the same time, the oligofectamine was diluted in 15 µl of Opti-MEM® I medium and incubated for 15 minutes at room temperature. After the incubation, the diluted siRNA was combined with the diluted oligofectamine (total volume is 200 µl) and incubated for 30 minutes at room temperature to allow the siRNA:oligofectamine complexes to form. The mixtures were then applied to the cells in a final volume of Opti-MEM® I medium giving a final siRNA concentration of 50 nM.

After 4 hours incubation at 37℃ in a 5% CO_2 humidified atmosphere, a solution 3X FBS medium was added to each well.

12. Protein expression & purification

Recombinant GST fusion proteins were expressed in *E. coli* BL21-CodonPlus-RIL (Stratagene, Wilmington, DE, USA) Recombinant GST-fusion proteins were induced in *E.coli* BL-21 strain with 0.3 mM IPTG for 3 hours at room temperature. After centrifugation cells were lysed with lysis buffer according to their solubility and the supernatant was directly used for binding to glutathione Sepharose 4B beads 50% slurry (Sigma). After overnight of binding, washes were performed in Hunt wash buffer (20 mM Tris-HCl pH8; 100 mM NaCl; 1 mM EDTA; 0.5% NP-40; 100 µg/ml PMSF; 1 µg/ml leupeptin; 1 µg/ml aprotin) and in PBS 1X and GST Fusion Protein were purified with B-PER GST Fusion Protein Spin Purification Kit (Pierce,

Rockford, IL,USA). Protein concentrations of the different elution fractions were measured using a protein assay reagent (Bio-Rad) using BSA as standard and analyzed by SDS-PAGE.

13. Protein analysis & turnover

Tumor cell types (6-7x10^7) were fractionated in mitochondrial and cytosolic extracts using the ApoAlert Cell Fractionation Kit (BD Biosciences, Clontech, Mountain View, CA, USA), according to the supplier's instructions. The isolated cytosolic fraction was further centrifuged at 10,000 *g* for 25 minutes at 4℃, and the supernatant was collected. Alternat ively, mitochondrial extracts were prepared from cell typers (5 × 10^7) washed in TD buffer (135 mM NaCl, 5 mM KCl, 25 mM Tris-Cl, pH 7.6) and allowed to swell for 10 minutes in ice-cold hypotonic CaRSB buffer (10 mM NaCl, 1.5 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5, plus protease inhibitors). Cells were Dounce-homogenized with 70 strokes, with addition of MS buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris, pH 7.6) to stabilize mitochondria (2 ml of 2.5× per 3 ml of homogenate). After removal of nuclear contaminants by centrifugation at 600 *g* for 15 minutes on ice, the supernatants were layered over a 1- to 2-M sucrose step gradient (10 mM Tris, 5 mM EDTA, pH 7.6, 2 mM DTT, plus protease inhibitors) and centrifuged at 110,000 *g* for 30 minutes at 4℃. Mitochondria were collected at the 1- to 1.5-M interphase by lateral suction through the tube, washed in 4 volumes of MS buffer at 9,400 *g*, and suspended in a final volume of 200 µl of MS buffer. The top layer,

containing a cytosolic and a free protein fraction, was collected and used in parallel experiments. The purity of mitochondrial fractions was examined by immunoblotting with antibody to mitochondrial COX IV (Cell Signaling).

Nuclear and cytosolic fractions were extracted using CelLytic NuCLEAR ExtractionKit (Sigma) according to the manufacture's instructions. The purity of nuclear fractions was evaluated by immunoblotting with RCC1 antibody (Santa Cruz).

Pull down experiments with recombinant fusion proteins (10 μg) were carried out. Total extracts from LN229 cells were incubated with IgG or an antibody to Chk2 or Hsp60 and the immune complexes were precipitated by the addition of protein A-Sepharose beads (Amersham) in 50 mm Tris, pH 7.5, 0.1% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 50 mm NaCl, 1 mm protease inhibitors (Roche Applied Science, Indianapolis, IN, USA), and 1 mm Na_3Vo_4. After washing, pellets or supernatants were separated by SDS-gel electrophoresis and analyzed by Western blotting.

For protein stability, HCT116 cells at 60% confluency were transfected with cDNAs encoding control plasmid, WT survivin, survivin ΔEx3 or survivin ΔEx3 carrying Ala substitutions in the three Chk2 phosphorylation sites (survivin-ΔEx3-Mut3), by lipofectamine. After 24 h, transfected cells were treated with 0.1 mg/ml cycloheximide(Sigma), harvested after 0, 2, 4, and 6 h, and total cell extracts were analyzed by Western blotting.

14. Western Blot Analysis

Equivalent amounts of cellular extracts fractionated, were blotted onto solid support and immunoassayed to qualitatively evaluate the expression of the proteins of interest.

14.1. SDS-Polyacrylamide Gel Electrophoresis

Samples were separated on 5% or 12% SDS polyacrylamide gels.

An aliquot of cellular extracts containing 40 µg of proteins were mixed in a 0.5 conical tube with an equal volume of 2x SDS loading buffer (100 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue (Sigma), 20 (v/v) glycerol and 200 mM DTT). Tubes were heated at 100℃ for 5 minutes, chilled on ice for an additional 5 minutes, centrifuged at 4℃ at 12,000 rpm for 5 seconds and loaded onto the SDS-PAGE system.

Proteins were fractionated running the gel at constant voltage (150 volt) by means of a Biorad 200/2.0 power supply (Biorad). The run was stopped when the 6.8 KDa band of the colour marker reached the bottom of the gel.

14.2. Transfer of Fractionated Proteins into Nitrocellulose Filter

Protein blotting was performed in an electrophoresis protein transfer unit (Biorad). When the SDS-PAGE was approaching the end of its run, one piece of Immobilon-PSQ membranes (Millipore) was cut and soaked in Methanol (Fisher Scientific). After 1 minute the membrane and four pieces of Whatman 3MM (Whatman International LtD., Maidstone, United Kingdom) and), were soaked for at least 5 minutes in transfer buffer [50 mM Tris base, 100 mM Glycine, 0.01% (w/v) SDS, 20% (v/v) methanol (Fisher Scientific), pH 7.5]. At the end of the run, the glass plates holding the SDS-polyacrylamide gel were removed from the electrophoresis tank, the glass plates were opened and, by means of a scalpel, the stacking gel was removed. The resolving gel was placed exactly on the top of the filter and then sandwiched between two pairs of transfer buffer wetted-Whatman 3MM papers. Possible air bubbles trapped were displaced by repeated rolling with a pipette tip. The sandwich was then placed between two transfer buffer wetted-porous pads and two plastic supports and the entire construction was immersed in the electrophoresis transfer tank containing transfer buffer with the filter placed toward the anode. Transfer of the protein from the gel to nitrocellulose membrane was carried out on ice for 1 hours at 0.35 amper (constant amperage) by means of a Biorad 200/2.0 power supply.

14.3. Immunological Detection of PVDF-Immobilised Proteins

At the end of the transfer, the PVDF filter was recovered from the sandwich and, in order to verify the correct protein blotting, it was stained for 5 minutes in a clean tray containing a Ponceau S red dye solution (Sigma) with gentle agitation at room temperature. When the bands of proteins were visible, the filter was destained with several washes of distilled water at room temperature. To completely destain the Ponceau S red dye bound to the proteins, nitrocellulose slices were washed three times for 20 minutes at room temperature on a shaking platform with blocking solution [5% (w/v) dried-nonfat milk, 0.1% (v/v) Tween-20 (Sigma) and PBS]; filter slices were then incubated at room temperature on a shaking platform in blocking solution to mask potential nonspecific antibodies binding sites and, thus, to reduce general background. After 1 hour, blocking solution was removed and filters were incubated over night at 4℃ on a shaki ng platform with the specific antibody-containing solution. Detection of proteins of interest was achieved by using polyclonal or monoclonal antibodies diluted in blocking solution. At the end of the over night incubation, primary antibodies were removed and filter slices were washed 3 times for 20 minutes with T-PBS wash solution [0.1% (v/v) Tween-20 in PBS] at room temperature on a shaking platform. PVDF filters were then incubated at room temperature on a shaking platform with the secondary anti-mouse or anti-rabbit Ig horseradish peroxidase-linked whole antibodies (Amersham) diluted 1:1000 in blocking solution. After a 1-hour hybridisation, secondary antibodies were removed and filter slices were washed three times for 20 minutes at room temperature

on a shaking platform with T-PBS wash solution followed by a rapid wash with PBS. Antibodies bound to proteins of interest on PVDF slices were detected by means of the enhanced chemoluminescence system (ECL) (Amersham) according to the manufacturer's instructions using Kodak Bio Max Film (Kodak, Sigma).

15. Kinase assays

Recombinant proteins were incubated with human active Chk2 (R&D Systems, 0.1 µg/µl) in kinase buffer (pH 7.2) containing 25 mM MOPS, 12.5 mM β-glycerolphosphate, 25 mM MgCl2, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT, and 0.1 mM ATP in the presence or absence of 32P-γATP (Amersham) for 20 min at 30℃. Recombinant Cdc25 was used as a control Chk2 substrate. In control experiments, Chk2 was omitted from the kinase reaction. At the end of the incubation, the various samples were separated by SDS gel electrophoresis, and phosphorylated bands were detected by autoradiography. Equal protein loading under the various conditions was confirmed by Coomassie blue staining of the gel.

16. Flow cytometry analysis

Tumor cell types were treated with vehicle or the DNA-damaging agent etoposide (2.5 μM) or camptothecin (CPT, 2.5 μM) for 16 h at 37℃, fixed in 70% ethanol for 30 min and then incubated for 16 h with a solution of propidium iodide (PI) in PBS, pH 7.4.

Cells were analyzed for DNA content by flow cytometry with collection of 10,000 events per sample, and analysis using FlowJo software (Tree Star, Ashland, OR, USA). For detection of γH2AX, HCT116 cells were transfected with control vector, WT survivin, survivin-ΔEx3 or Chk2 phosphorylation-defective survivin-ΔEx3-Mut3, treated with etoposide or CPT (1.25-2.5 μM) for 12 h, and fixed in 1% paraformaldehyde for 15 min and 70% ethanol for 30 min. After permeabilization with a solution of 0.2% Triton X-100 plus 1% BSA in PBS, pH 7.4 for 10 min, cells (1x106) were incubated with a primary antibody to γH2AX for 90 min followed by Alexa Fluor® 488 secondary antibody for additional 60 min. Cells were washed, stained with PI and analyzed by multiparametric flow cytometry. Ten thousand events were collected and analyzed using FlowJo software. Data on *y*-axis represent the levels of γH2AX expressed as arbitrary fluorescence units.

17. Homologous double strand DNA break reapir assay

A pDR-GFP plasmid that utilizes two modified GFP genes (I-Sce1-GFP and iGFP) to create a recombination reporter was used. Transfection of the I-Sce1 endonuclease in cells stably expressing pDR-GFP results in DSB at the position of the I-Sce1 site, whose repair by a non-crossover gene conversion with iGFP reconstitutes a functional GFP gene. Cells were analyzed by flow cytometry on a green (FL1) versus orange (FL2) fluorescence plot with 20% or 25% orange minus green compensation to aid in the detection of faint positives. GFP-negative cells possess autofluorescence and fall along the green/orange diagonal, whereas GFP+ cells appear in a separate population shifted off the diagonal of cells and toward FL1. Stable clones of HCT116-pDR-GFP cells were transfected with pCMV-I-SceI alone or together with WT survivin, survivin-ΔEx3 or survivin ΔEx3-Mut3 cDNA. After 3 d, cells ($2\text{-}5x10^4$) were trypsinized, suspended in PBS, pH 7.4, containing 0.5% FBS, and analyzed by multiparametric flow cytometry with quantification of the percentage of green fluorescent cells relative to the total cell number.

18. Soft agar colony formation

HCT116 cells ($5x10^5$) were transfected with pcDNA, survivin-ΔEx3 or survivin-ΔEx3-Mut3, treated with or without etoposide (1.25 μM), and suspended in 1.5 ml of DMEM supplemented with 10% FBS and 0.35% bactoagar (Becton Dickinson, Sparks, MD) in 36 mm tissue culture plates

containing 1.5 ml of 0.75% agarose in growth medium at the bottom layer. Plates were incubated at 37°C in a 5% CO2 incubator for 2 weeks. Colonies were stained with 0.005% crystal violet (Sigma), visualized by phase contrast microscopy, and counted using a dissecting microscope under high-power field. The size of colonies (≤100 μm or 100-200 μm) was quantified using NIH Image J software.

Experiments were repeated twice with similar results.

19. Histology

Primary human tissue samples devoid of all patient identifiers were obtained anonymously after informed consent from the Department of General and Gastroenterological Surgery at Osaka Medical College. Specimens were obtained from patients undergoing surgical or endoscopic resections, and were representative of different stages of the adenoma-tocarcinoma transition of the colonic epithelium. A histologic diagnosis of normal colon, hyperplasia, moderate or severe dysplasia, or adenocarcinoma was obtained for each sample by independent pathological review of hematoxylin-eosin (H&E)-stained tissue slides. Samples were processed for antigen retrieval, as described (20), and analyzed for expression of survivin, Chk2, Thr68-phosphorylated Chk2 or p53, by immunohistochemistry. Twenty cases per each condition were scored, and a cutoff of 5% positively stained cells was used.

20. Statistical analysis

Data were analyzed using the unpaired *t* test on a GraphPad software package for Windows (Prism 4.0). All tests on gene expression analysis of survivin-ΔEx3 were done using samples for each GEO dataset separately. Differences in survivin-ΔEx3 gene expression in datasets with paired cancer/control samples (datasets 3 and 4) were tested using the two-tail *t*-test. Additional comparisons were done to estimate the significance of association of survivin-ΔEx3 expression with available annotation data using the Spearman correlation test. The level of significance was set at $p<0.05$

Chapter 4

RESULTS

1. DIFFERENTIAL EXPRESSION OF SURVIVIN-ΔEX3 IN HUMAN CANCER

We began this study by taking an unbiased bioinformatics approach to examine the expression of survivin-ΔEx3 in 14 publicly available cancer-associated GEO datasets with gene expression profiles assayed by Affymetrix human exon arrays (Table II). A schematic diagram of the genomic organization of the *survivin* gene, and its relationship to the probesets on the arrays are shown in Figure 17A. By principal component analysis (Figure 18A), the expression of probeset 9 (deleted in survivin-ΔEx3) across samples differed from that of other 8 probesets common to all the survivin isoforms, demonstrating the ability of the microarray platform to distinguish between survivin isoforms that either contain or lack exon 3 (Figure 18A). A hierarchical clustering based on differential probeset expression patterns is shown in Figure 18B. Given the average expression of probesets 4, 5, 13, 14, 15 and 17 (***X*** = cumulative expression of all three isoforms) and probeset 9 (***Y*** = expression of survivin and survivin-2B isoform, but not survivin-ΔEx3), we determine that ***Y***<***X*** for the majority of samples, indicating an expected relation between exon expression (Figure 17B). The specific expression of survivin-ΔEx3 was then calculated as the difference ***X***-***Y***, and then log2 transformed for further investigation. By this analysis, survivin-ΔEx3 mRNA was found to be expressed at significantly higher levels in tumors of the ovary, stomach, brain, lung and prostate, as compared to matched normal tissues (Figure 17C). When analyzed for a potential

relationship with disease progression, survivin-ΔEx3 levels were elevated in grade IV glioblastoma compared to low grade oligodendrogliomas, in metastatic compared to localized prostate cancer, and in stage 4 compared to stage 1 neuroblastoma (Figure 17D and Table III). Consistent with these findings, increased expression of survivin-ΔEx3 also correlated with poorer survival (p=0.01, spearman correlation), greater incidence of recurrence (p=6x10-5, spearman correlation) and advanced Gleason grade (p=0.0004) in prostate cancer, N-myc amplification in neuroblastoma, H.pylori infection in gastric cancer, and chromosome 11q13 amplification in squamous head and neck cancer (Table III).

Table II. GEO mRNA datasets used in the study

#	GEO acc	Study Set	Tot	Control	Cancer
1	GSE 9385	Identification of differentially regulated splice variants and novel exons in glial brain tumor using exon arrays (PMID: 17575129)	55	6	49
2	GSE 11967	Identifying alternative hyper-splicing signatures in MG-thymoma by exon arrays (PMID: 18545673)	4	2	2
3	GSE 12236	Whole Genome Exon Arrays Identify differential Expression of Alternatively Spliced, Cancer-related Genes in Lung Cancer (PMID: 18927117)	40	20	20
4	GSE 13195	Gene expression and alternative splicing in human gastric cancer	50	25	25
5	GSE 21034	Whole-transcript and exon-level expression data for human primary and metastatic prostate cancer samples and control normal adjacent benign prostate (PMID: 20579941)	179	29	150
6	GSE 21440	Gene expression analysis of pancreatic cancer associated fibroblasts (PMID: 20215540)	12	3	9
7	GSE 23361	Significant Downregulation of Platelet Gene Expression in Metastatic Lung Cancer (PMID: 21500395)	12	7	5
8	GSE 29156	Serous ovarian benign tumor and type II carcinoma data set for expression and paracrine signaling investigation	39	31	8
9	GSE 29301	Exon-Level Expression Data from Primary Human B Cells and Lymphoblastoid Cell Lines (LCLs) on HuEx and U133 2.0 plus Platforms	8	4	4
10	GSE 16534	Exon array profiling detects EML4-ALK fusion in breast, colorectal and non-small cell lung cancers. (PMID: 19737969)	153	na	153
11	GSE 27388	Exon-array profiling of squamous cell carcinoma and adenocarcinoma in human cervical FFPE samples (PMID:21407225)	28	na	28
12	GSE 27501	Quantitative Analysis of Alternative Spliced Variants in HNSCC (PMID: 21745820)	15	na	15
13	GSE 27608	Exon array analysis reveals neuroblastoma tumors have distinct alternative splicing patterns according to stage and MYCN amplification status (PMID: 21501490)	47	na	47
14	GSE 29682	Comparison between cell lines from 9 different cancer tissue (NCI-60)	60	na	60

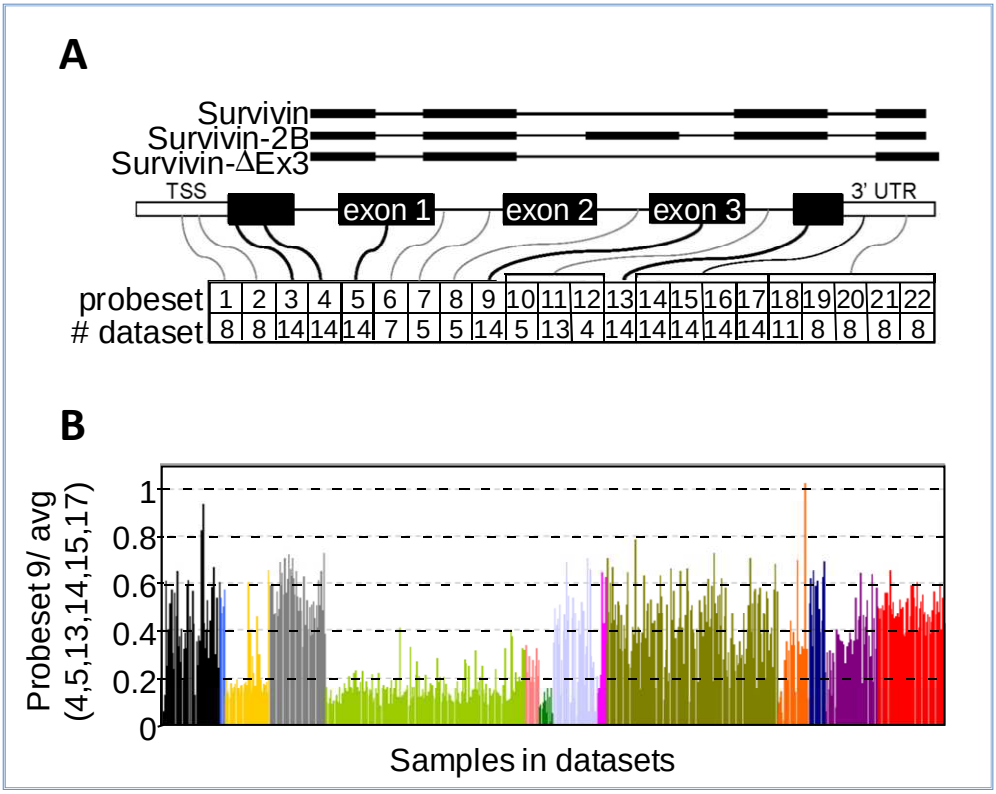

Figure 17A-B. Genome-wide bioinformatics analysis of survivin-ΔEx3 expression in cancer.

A. Schematic diagram of genomic organization of WT survivin, spliced variants survivin-2B and -ΔEx3 (top), and distribution of probesets and datasets with significant expression (bottom).
B. Ratio between probeset 9 targeting NM_001168 (WT survivin) and NM_001012271 (survivin-2B), and average expression of probesets targeting all surviving isoforms (expected ratio is <1). The different colors indicate samples from 14 different datasets, incremental from the 1st dataset on the left to the 14th dataset on the right.

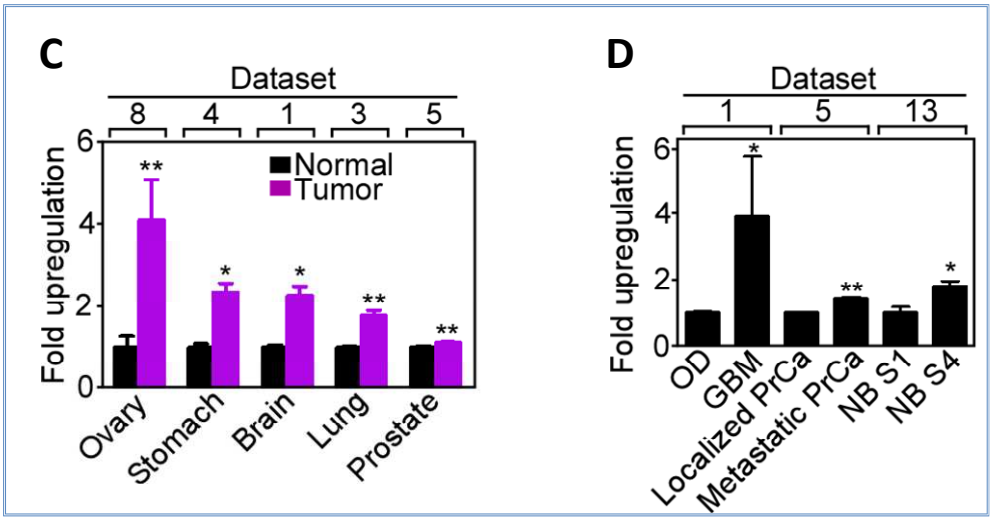

Figure 17C-D. Genome-wide bioinformatics analysis of survivin-ΔEx3 expression in cancer.

C. Fold upregulation of survivin-ΔEx3 expression in the indicated tumor types relative to matched normal tissues within each dataset. The number of cases in the normal (N) or tumor (T) group is as follows: ovary (N, n=31; T, n=8); stomach (N, n=25; T, n=25); brain (N, n=6; T, n=49); lung (N, n=20; T, n=20); prostate (N, n=29; T, n=150); thymus (N, n=2; T, n=2). Mean±SEM.

D. Increased expression of survivin-ΔEx3 during tumor progression. OD, oligodendroglioma; GBM, grade IV glioblastoma; PrCa, prostate cancer; NB, neuroblastoma. The number of cases per each condition is as follows: OD, n=2; GBM, n=28; localized PrCa, n=131; metastatic PrCa, n=19; stage (S) 1 NB, n=10; stage (S) 4 NB, n=37. For panels C and D, the dataset examined per each condition is indicated. Mean±SEM.

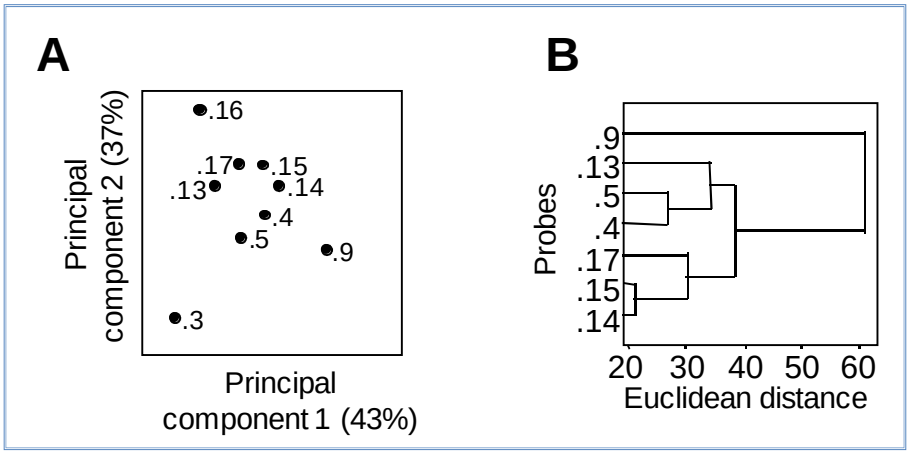

Figure 18. GPL5188 platform probeset .

A. Relationship between all probeset expression profiles.
B. Hierarchical clustering of expression profiles of probesets retained for analysis.

Table III. Tissue expreession of survivin-ΔEx3.

Set	Cancer	Comparison(group1/group2)	n1	n2	Test	P	Value
1	Brain	glioblastoma / low grade oligodendroglioma	28	2	t-test	0.004	fc=3.42
4	Gastric	Helicobacter pylori test yes/no	14	9	t-test	0.005	fc=1.86
5	Prostate	Metastatic/primary	19	131	t-test	$9x10^{-12}$	fc=1.42
5	Prostate	Death from prostate cancer	140		spearman	0.01	rho=0.21
5	Prostate	Biochemical recurrence (BCR)	140		spearman	$6x10^{-5}$	rho=0.33
5	Prostate	Pathological stage	141		spearman	0.02	rho=0.19
5	Prostate	Gleason grade	149		spearman	0.0004	rho=0.29
12	HNSCC	11q13 amplification yes/no	8	7	t-test	0.04	fc=1.81
13	NB	MYCN amplification yes/no	9	38	t-test	0.02	fc=1.75
14	Renal	Ploidy of the cell line	8		spearman	0.005	rho=0.88

HNSCC= HEAD AND NECK SQUAMOUS CELL CARCINOMA, NB=NEUROBLASTOMA, N 1=NUMBER OF SAMPLES IN GROUP 1, N 2=NUMBER OF SAMPLES IN GROUP 2, *FC*=UPREGULATION FOLD, *RHO*=SPEARMAN CORRELATION COEFFICIENT.

2. SUBCELLULAR LOCALIZATION OF SURVIVIN-ΔEX3

Transfection of FLAG-tagged survivin-ΔEx3 resulted in an exclusively nuclear localization in disparate tumor types, including HCT116, H460 or MDA-MB-431 cells (Figure 19A). In contrast, WT survivin was ubiquitously distributed in nuclear and cytosolic compartments in tumor cells [Fortugno *et al.*, 2002]. In addition, survivin-ΔEx3 was excluded from mitochondria of transfected MDA-MB-431 or H460 cells (Figure 19B).

Accordingly, treatment of H460 cells with a cell death stimulus, staurosporine (STS) caused loss of mitochondrial transmembrane potential, in a response unaffected by transfection of control plasmid or survivin-ΔEx3 (Figure 20).

To begin probing the function of survivin-ΔEx3 in tumor cells, we next designed isoform-specific siRNA oligonucleotides that selectively ablate the expression of endogenous survivin-ΔEx3 in tumor cells. Transfection of MCF-7 cells that constitutively express WT survivin [Dohi et al., 2007] with control, non-targeting siRNA did not significantly affect endogenous mRNA expression of survivin or survivin-ΔEx3, by RT-PCR (Figure 21, top). Conversely, transfection of isoform-specific siRNA reduced the expression of survivin-ΔEx3, whereas the levels of WT survivin were not affected (Figure 21, bottom). Under these conditions, siRNA silencing of surviving or survivin-ΔEx3 produced comparable defects in cell cycle progression, characterized by arrest at G2/M, and appearance of a cell population with >4N DNA

content, indicative of DNA endoreduplication (Figure 21, bottom) (Beltrami *et al.*, 2004).

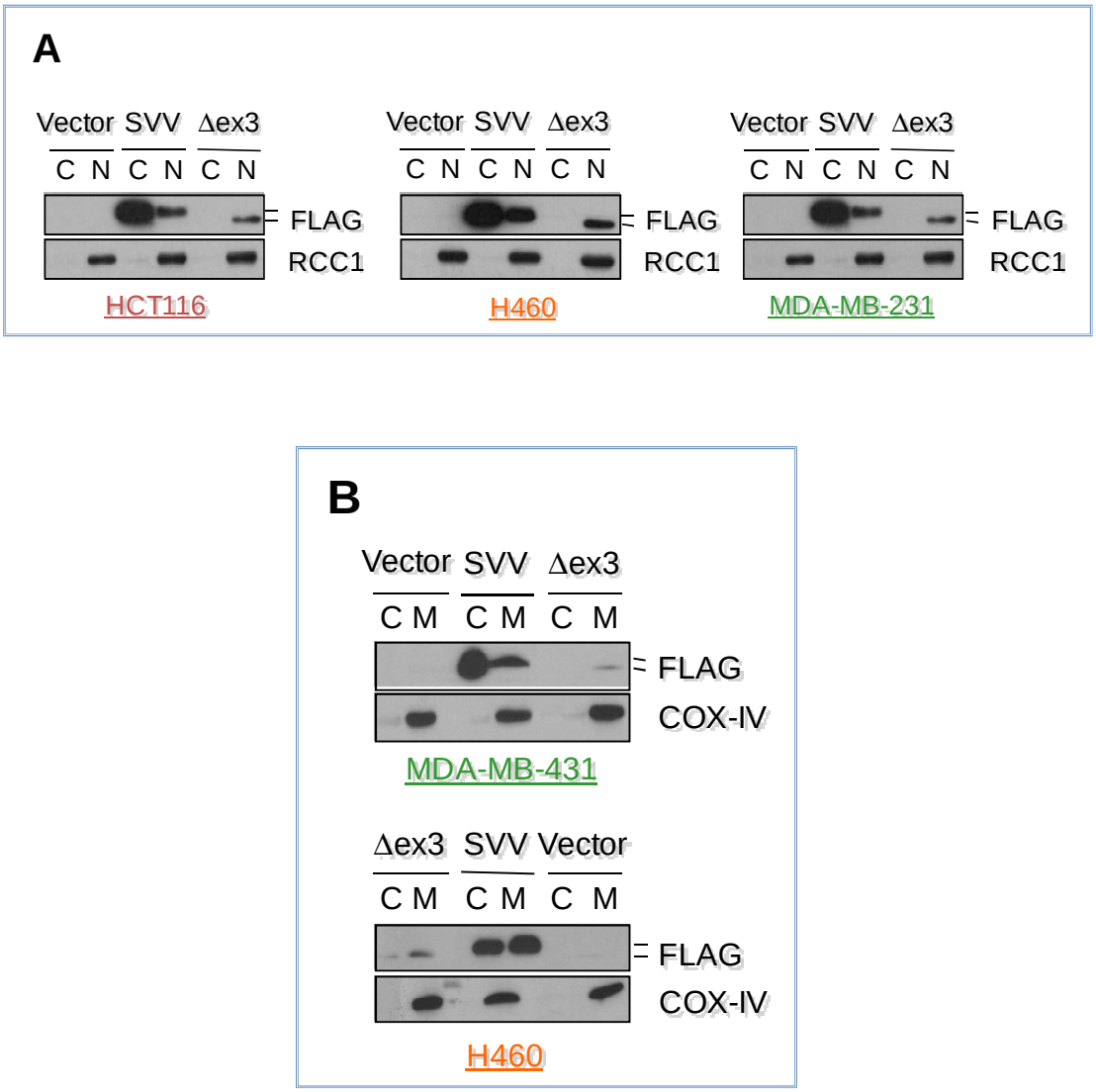

Figure 19. Nuclear localization of survivin-ΔEx3 in cancer.

A. The indicated tumor cell types were transfected with vector (pcDNA), FLAG-survivin (SVV) or FLAG-survivin-ΔEx3 cDNA, and isolated cytosolic (C) or nuclear (N) fractions were analyzed with an antibody to FLAG by Western blotting. RCC1 was used as a nuclear marker.

B. The indicated tumor cell types were transfected as in **A**, and isolated cytosolic (C) or mitochondrial (M) fractions were analyzed with an antibody to FLAG by Western blotting. COX-IV was used as a mitochondrial marker.

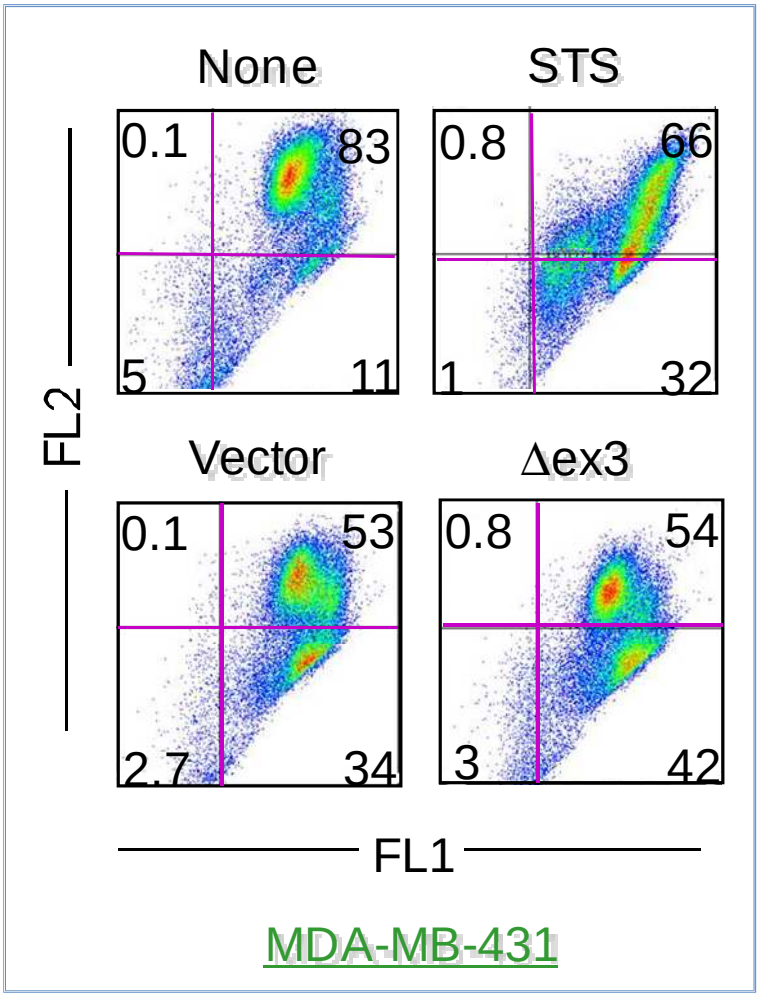

Figure 20. Mitochondrial transmembrane potential changes.

MDA-431 cells were transfected with vector (pcDNA) or survivin-ΔEx3 cDNA, treated with staurosporine (STS), and analyzed for changes in mitochondrial membrane potential by JC-1 staining and multiparametric flow cytometry. The data are expressed as ratio between green (FL1)/red (FL2) fluorescence. In each quadrant is indicated the percentage of cells.

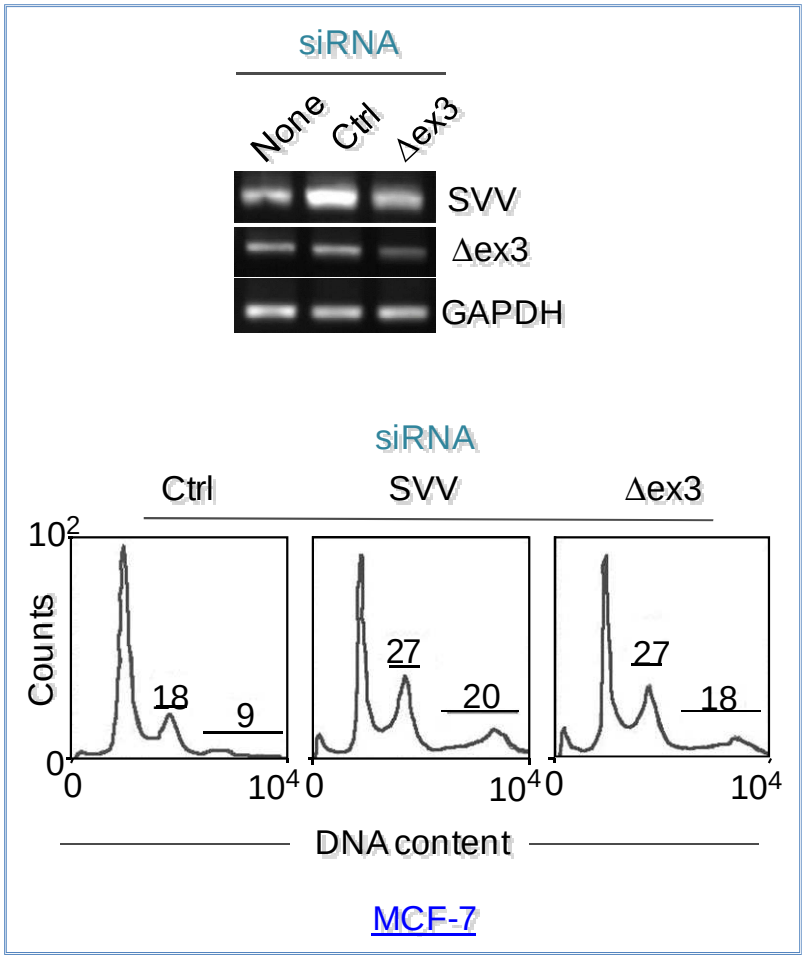

Figure 21. RT-PCR and DNA content after siRNA transfection.

MCF-7cells stably transfected with survivin were left untreated (None) or transfected with control (Ctrl) non-targeting or survivin- ΔEx3-directed siRNA and analyzed by RT-PCR (*top*) or DNA content by propidium iodide (PI) staining and flow cytometry (*bottom*). The percentage of cells in the G2/M or >G2/M fraction is indicated.

3. CHK2 PHOSPHORYLATION OF SURVIVIN-ΔEX3

By sequence analysis, three potential phosphorylation sites matching the consensus for the DNA damage checkpoint kinase, Chk2 (Reinhardt & Yaffe, 2009) were identified at Thr79, Ser98 and Thr127 in the unique -COOH terminus sequence of survivin-ΔEx3 (Figure 22A) (Li, 2005, Sampath & Pelus, 2007). In contrast, no putative Chk2 phosphorylation sites were identified in WT survivin. Consistent with these predictions, purified, active Chk2 readily phosphorylated recombinant survivin-ΔEx3 in a kinase assay, whereas WT survivin was not phosphorylated by Chk2 (Figure 22B). As control, Chk2 also phosphorylated its known substrate, Cdc25 (Figure 22B). Ala substitution of Thr127 in survivin-ΔEx3 (ΔEx3-Mut1), or double mutation of Thr79 and Ser98 (ΔEx3-Mut2) partially reduced phosphorylation by Chk2 (Figure 22A). Conversely, Ala mutagenesis of all three putative phosphorylation sites in survivin-ΔEx3, including Thr127/Thr79/Ser98 (ΔEx3-Mut3) abolished Chk2 phosphorylation in a kinase assay (Figure 22B).

A potential regulation of survivin-ΔEx3 by Chk2 phosphorylation was next investigated. When expressed in HCT116 cells, survivin-ΔEx3 exhibited considerably accelerated protein degradation compared to WT survivin, with an estimated half-life of 2h, as opposed to 6h for the main survivin isoform, by clycloheximide block and release experiments (Figure 23). In contrast, a Chk2 phosphorylation-defective survivin-ΔEx3 mutant (survivin-ΔEx3-Mut3) was considerably more stable in HCT116 transfectants, with an approximate half-life of 4h (Figure 23). In these experiments, expression of survivin-ΔEx3

or survivin-ΔEx3-Mut3 did not significantly affect total Chk2 levels in transfected HCT116 cells (Figure 23). Consistent with a rapid turnover of survivin-ΔEx3 in tumor cells, pellets of LN229 cell extracts precipitated with recombinant GST-survivin or GST-survivin-ΔEx3 did not contain co-associated Chk2, by pull down experiments and Western blotting (Figure 24, top). Conversely, recombinant survivin, but not survivin-ΔEx3 associated with the mitochondrial chaperone, Hsp60 (Figure 24, bottom), in agreement with previous observations (Ghosh *et al.*, 2008).

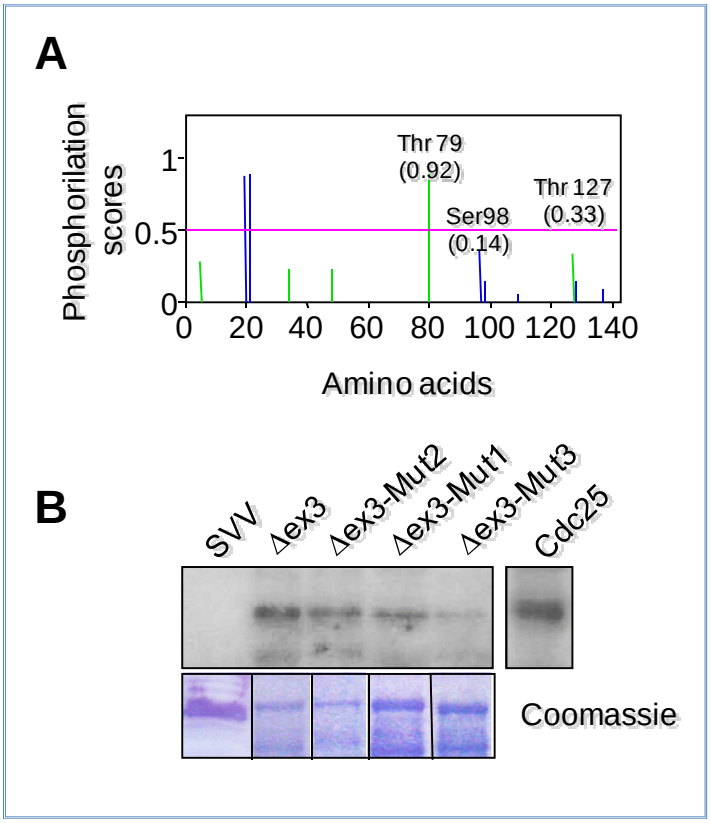

Figure 22. Chk2 phosphorylation of survivin-ΔEx3.

A. Indication of the position of three putative consensus phosphorylation sites for Chk2 at Thr79, Ser98 and Thr127 in the unique -COOH terminus sequence of survivin-ΔEx3. **B**. Recombinant active Chk2 was incubated with recombinant survivin (SVV), survivin-ΔEx3 or survivin-ΔEx3 Thr127→Ala (Mut1), Thr79/Ser98→Ala (Mut2) or Thr127/Thr79/Ser98→Ala (Mut3) in a kinase assay, and radioactive bands were visualized by autoradiography. Recombinant Cdc25 was used as a control for a Chk2 substrate. *Bottom*, Coomassie blue staining of recombinant proteins used inthe kinase assay.

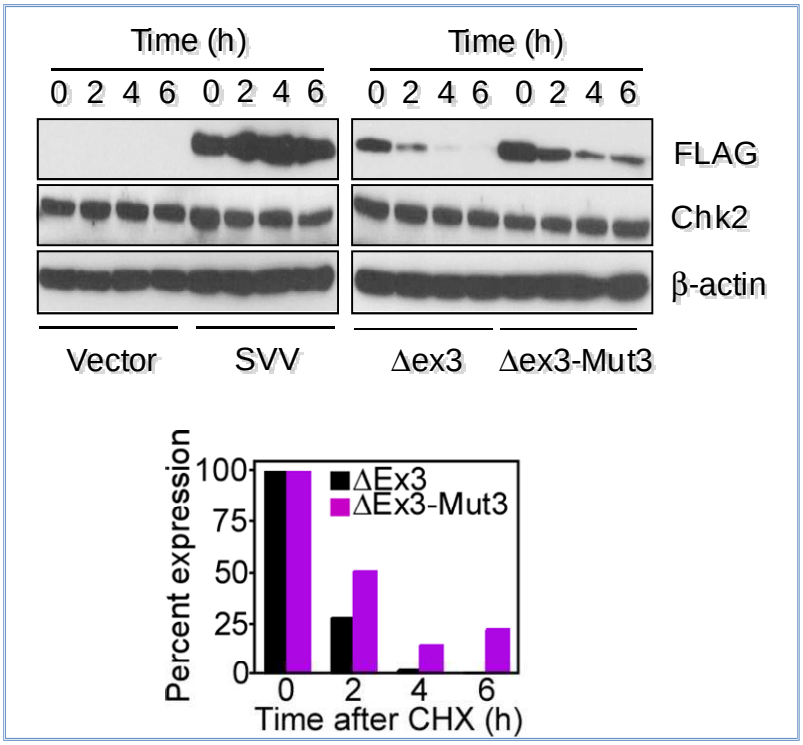

Figure 23. Survivin expression and stability after cycloheximide treatment.

HCT116 cells were transfected with the indicated plasmids, treated with cycloheximide, harvested at the indicated time intervals after release and analyzed by Western blotting.
Bottom, densitometric quantification of protein bands. Similar results were obtained in an independent experiment: survivin-ΔEx3, 2 h, 61.2%; 4 h, 40.6%; 6 h, 3.6%; survivin-ΔEx3-Mut3, 2 h 58.1%; 4 h, 48.7; 6 h, 25.7%.

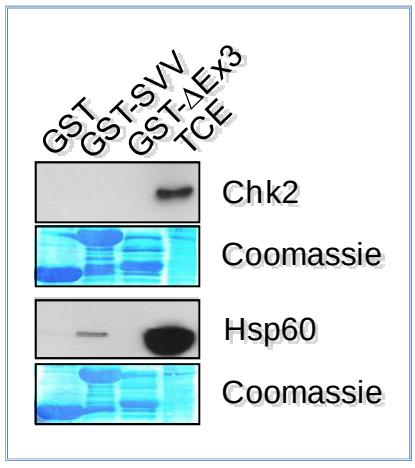

Figure 24. Pull down experiments and Western Blotting.

Recombinant GST, GST-survivin (SVV) or GST, survivin-ΔEx3 were incubated with LN229 total cell extracts (TCE) and bound proteins were analyzed with an antibody to Chk2 (*top*) or Hsp60 (*bottom*) by Western blotting. Recombinant proteins used for the pull down experiments were stained with Coomassie blue (*bottom panels*)
.

4. PARTICIPATION OF SURVIVIN-ΔEX3 IN A DNA DAMAGE-SENSING CHECKPOINT IN TUMOR CELLS

Based on these results, we next asked whether survivin-ΔEx3 contributed to Chk2 regulation of the DNA damage response (Jeggo & Lobrich, 2007), and we treated HCT116 cells with the topoisomerase II inhibitor and inducer of double strand DNA breaks (DSB), etoposide. DNA damage induction under these conditions resulted in the appearance of Ser139-phosphorylated histone H2AX, or γH2AX (Bonner *et al.*, 2008), a marker of unrepaired DNA damage (Jeggo & Lobrich, 2007), in a reaction that peaked 16 h after treatment (Figure 25A,B). Transfection of HCT116 cells with WT survivin or survivin-ΔEx3 did not affect the kinetics or magnitude of γH2AX reactivity after etoposide treatment (Figure 25A,B). In contrast, expression of Chk2 phosphorylation-defective survivin-ΔEx3-Mut3 abolished γH2AX reactivity in response to etoposide (Figure 25A,B).

To test the specificity of γH2AX reactivity by survivin-ΔEx3, we next treated HCT116 cells with the topoisomerase I inhibitor, camptothecin (CPT), which induces single strand DNA breaks. CPT induced robust and time-dependent γH2AX reactivity in HCT116 cells transfected with control vector (Figure 25C). Differently from the results obtained with etoposide, transfection of HCT116 cells with WT survivin, survivin-ΔEx3 or survivin-ΔEx3-Mut3 did not reduce γH2AX reactivity in response to CPT treatment (Figure 25C).

Second, we asked whether Chk2 phosphorylation of survivin-ΔEx3 affected homologousdirected DSB repair. For these experiments, we used

HCT116 cells stably transfected with two modified GFP genes that create a GFP-DR recombination reporter (Pierce *et al.*,1999, Moynahan *et al.*, 2001). Co-transfection of HCT-pDR-GFP cells with pCMV-I-SceI and control vector caused the appearance of GFP+ cells indicative of homologous DSB repair (Figure 26) (Pierce *et al.*,1999, Moynahan *et al.*, 2001). Co-transfection of WT survivin, survivin-ΔEx3 or survivin-ΔEx3-Mut3 cDNA together with pCMV-I-SceI did not significantly affect homologous DSB repair (Figure 26). As control, no GFP+ cells were detected in the absence of pCMV-I-SceI (Figure 26), confirming that spontaneous intrachromosomal DNA conversion was negligible with this assay (Pierce *et al.*,1999, Moynahan *et al.*, 2001).

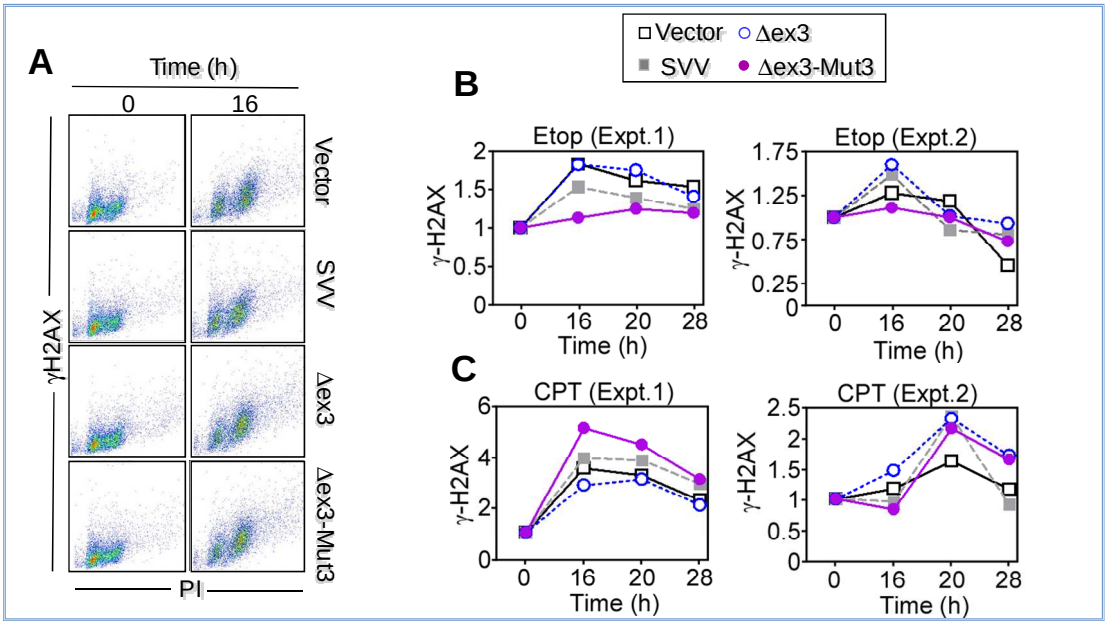

Figure 25. Survivin-ΔEx3 regulation of a DNA damage sensing checkpoint.

A. HCT116 cells were transfected with vector (pcDNA), survivin (SVV), survivin-ΔEx3 or Chk2 phosphorylation-defective survivin-ΔEx3-Mut3 cDNA, treated with etoposide and analyzed after 16 h for γH2AX (*y*-axis) and PI (*x*-axis) staining by multiparametric flow cytometry. **B.** The experimental conditions are as in **A**, except that expression of γH2AX in transfected HCT116 cells was normalized at the indicated time intervals after etoposide treatment. **C.** HCT116 cells were transfected as in **A**, treated with camptothecin (CPT), and analyzed for γH2AX expression at the indicated time intervals. In the panels **B** and **C** are shown two independent experiments (Expt).

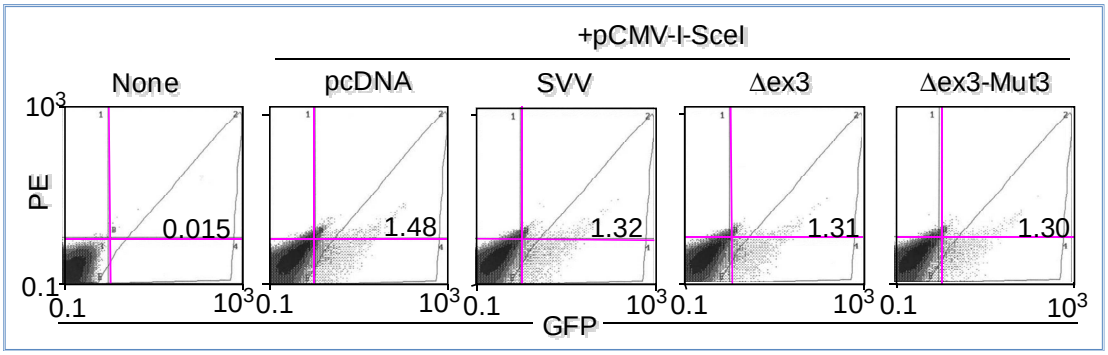

Figure 26. Homologous directed DSB repair.

HCT116 DR-GFP cells were transfected with pCMV-I-SceI without (None) or with pcDNA, survivin (SVV), survivin-ΔEx3 or survivin-ΔEx3-Mut3 cDNA, and analyzed for changes in GFP expression, by multiparametric flow cytometry. The percentage of GFP^+ cells under the various conditions tested is indicated. PE, phycoerythrin.

5. REQUIREMENTS OF SURVIVIN-ΔEX3 FOR A DNA DAMAGE-SENSING CHECKPOINT

A key regulator of the DNA damage-sensing pathway is the Mre11-Rad50-Nsb1 (MRN) complex (Takemura *et al.*, 2006), and its potential participation in modulation of γH2AX reactivity by survivin-ΔEx3 was next investigated. Transfection of survivin-ΔEx3 in colorectal adenocarcinoma SW480 cells, which contain normal levels of the MRN complex, as opposed to HCT116 cells (Takemura *et al.*, 2006), resulted in robust γH2AX expression in response to etoposide, by Western blotting (Figure 27A). In contrast, expression of survivin-ΔEx3-Mut3 nearly completely abolished γH2AX reactivity after DNA damage (Figure 27A).

We next asked whether Chk2 phosphorylation of survivin-ΔEx3 affected downstream pathway(s) of the DNA damage response. Similar to the data above, transfection of survivin-ΔEx3-Mut3 suppressed time-dependent γH2AX induction in etoposide-treated HCT116 cells, by Western blotting (Figure 27B). In contrast, etoposide-induced Ser15 phosphorylation of p53, increased levels of total p53 levels, or upregulation of the cyclin dependent kinase inhibitor, p21, were indistinguishable in HCT116 cells expressing survivin-ΔEx3 or survivin-ΔEx3-Mut3 (Figure 27B). Also, no differences were observed in the kinetics of etoposide-induced Chk2 phosphorylation on Thr68 in the presence of survivin-ΔEx3 or survivin-ΔEx3-Mut3 (Figure 27B).

We next asked whether defective γH2AX expression by survivin-ΔEx3-Mut3 affected clonogenic tumor cell survival. In the absence of etoposide,

transfection of HCT116 cells with survivin-ΔEx3 or survivin-ΔEx3-Mut3 significantly enhanced colony formation in soft agar, compared to control transfectants (Figure 28), suggesting a cytoprotective role of both molecules irrespective of DNA damage or Chk2 phosphorylation. After etoposide-induced DNA damage, expression of survivin-ΔEx3 still supported HCT116 colony formation (Figure 28). In contrast, transfection of survivin-ΔEx3-Mut3 preferentially impaired the ability of HCT116 cells to form larger colonies of up to 200 µm diameter after etoposide treatment (Figure 28).

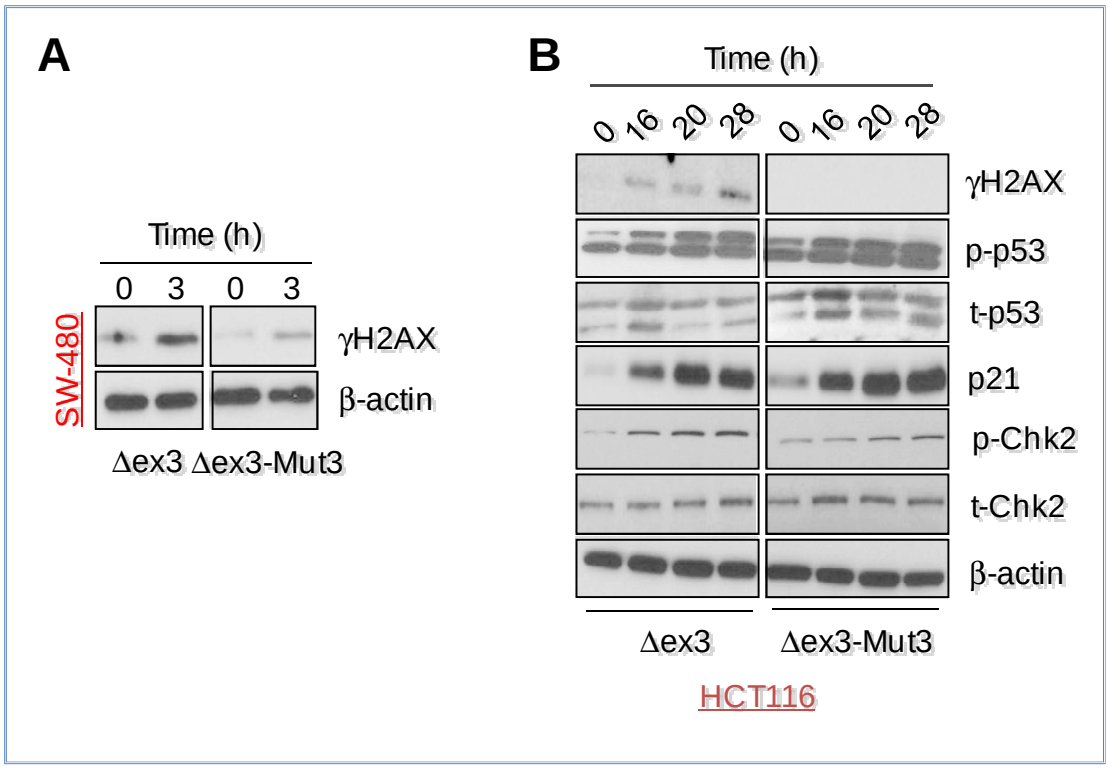

Figure 27. Requirements of survivin-ΔEx3 during the DNA damage response.

A.SW480 cells were transfected with survivin-ΔEx3 or Chk2 phosphorylation-defective survivin-ΔEx3-Mut3 cDNA, treated with etoposide and analyzed after the indicated time intervals, by Western blotting.

B. HCT116 cells were transfected with survivin-ΔEx3 or survivin-ΔEx3-Mut3 cDNA, treated with etoposide and analyzed at the indicated time intervals by Western blotting.

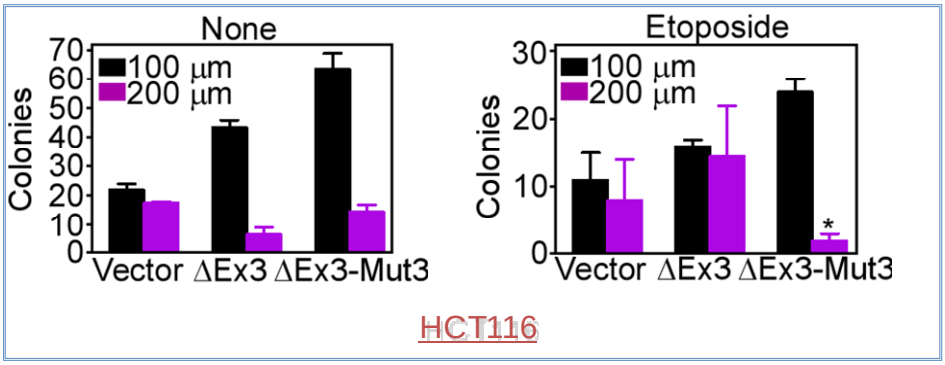

Figure 28. Clonogenic tumor cell survival.

HCT116 cells were transfected with vector (pcDNA), survivin-ΔEx3 or survivin-ΔEx3 cDNA, left untreated (None) or exposed to etoposide, and analyzed for colony formation in semisolid medium after 10 day. Colonies were imaged by phase contrast microscopy (magnification, x50), and quantified for diameters of 50-100 υm or 100-200 υm using Image J software. Mean±SEM of replicates from two independent experiments.

6. EXPRESSION OF ACTIVE CHK2 DURING STEPWISE TUMORIGENESIS IN HUMANS

Analysis of human tissue samples representative of the colorectal adenoma-to-carcinoma transition revealed that Thr68-phosphorylated Chk2 was abundantly expressed at the earliest changes of colonic hyperplasia, and throughout all stages of colorectal tumorigenesis, including moderate or severe dysplasia and adenocarcinoma (Figure 29A, B). In contrast, Thr68-phosphorylated Chk2 was undetectable in the normal colonic epithelium, and total Chk2 protein levels were comparable at all stages of colorectal tumorigenesis, as well as in the normal mucosa of the colon (Figure 29A, B). In addition, reactivity for survivin and p53 began to increase in dysplastic lesions of the colon, and their expression remained elevated in colorectal adenocarcinomas (Figure 29A, B).

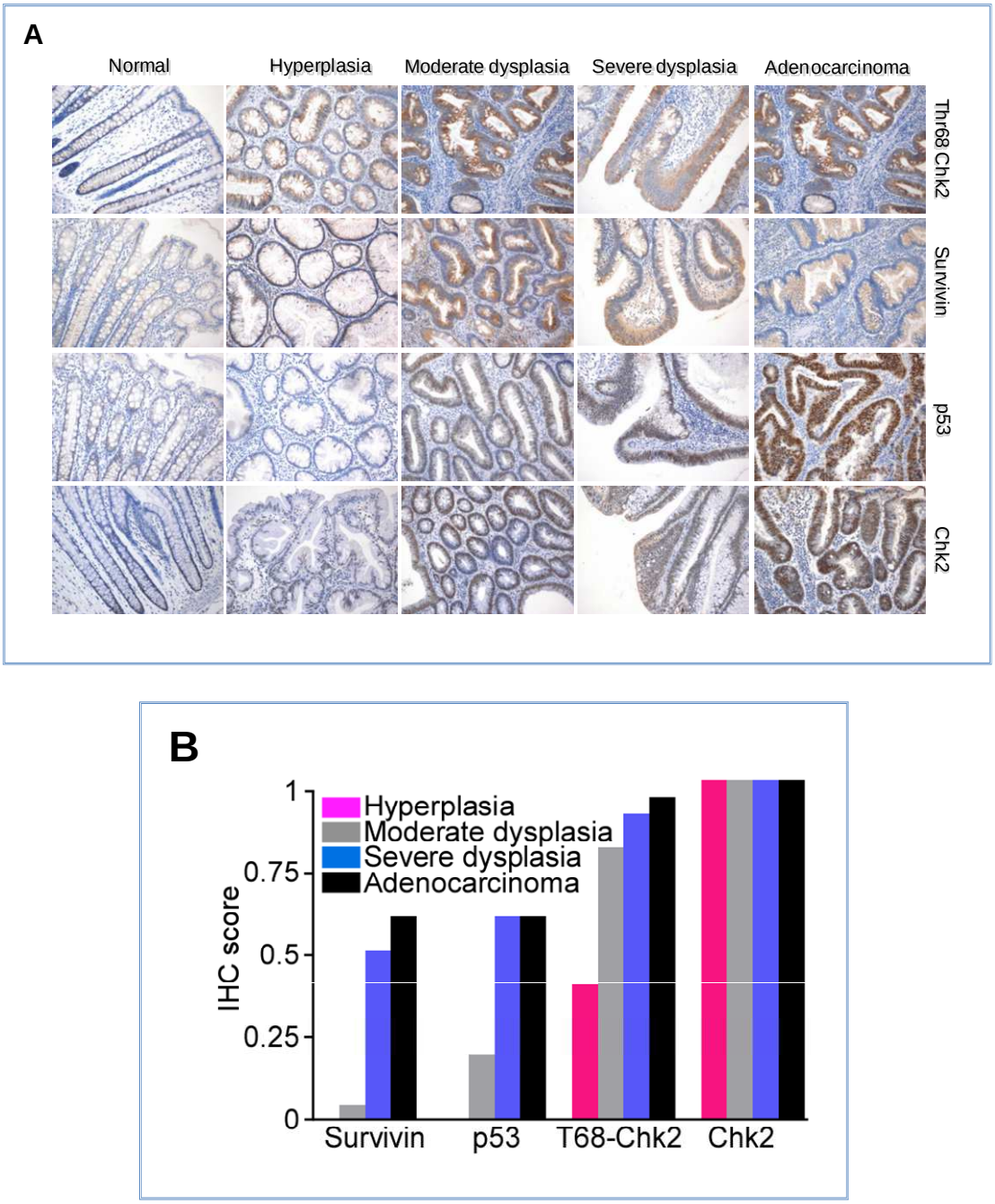

Figure 29. Chk2 activation during colorectal tumorigenesis, in vivo.

A. Patient-derived surgical or endoscopic specimens representative of progressive stages of the colorectal adenoma-to-carcinoma transition (normal mucosa, hyperplasia, moderate dysplasia, severe dysplasia and adenocarcinoma) were analyzed for expression of Thr68-phosphorylated Chk2, survivin, p53, or Chk2, by immunohistochemistry. **B.** Tissue slides representative of the indicated histologic diagnoses (20 cases/each) were scored for positively stained cells using a 5% cutoff.

Chapter 5

DISCUSSION

In this study, we have shown that survivin-ΔEx3, a survivin spliced variant with still largely elusive functions (Li, 2005, Sampath & Pelus, 2007), is a nuclear protein phosphorylated by the DNA damage checkpoint kinase, Chk2 (Reinhardt & Yaffe, 2009) on three novel amino acids located in its unique –COOH terminus region. Expression of non-phosphorylatable survivin-ΔEx3 exhibited enhanced stability in vivo, suppressed γH2AX reactivity specifically in response to DSB, and impaired long-term clonogenic tumor cell survival. When analyzed in human tumors, survivin-ΔEx3 was differentially expressed in the transformed cell population, correlating with advanced disease and other markers of unfavorable prognosis.

A potential involvement of the survivin pathway in the DNA damage response has emerged in recent studies. Although this was originally linked to the ability of survivinexpressing cells to counter radiation-induced apoptosis (Kanwar *et al.*, 2010), recent evidence suggested a more direct role for survivin in specific aspects of DNA damage and repair mechanisms, potentially independent of cytoprotection (Rodel *et al.*, 2011). Accordingly, siRNA or dominant negative targeting of survivin was associated with reduced DNA repair response(s), especially in gliomas (Chakravarti *et al.*, 2004), and exposure of tumor cells to YM155, a small molecule survivin inhibitor currently in the clinic (Giaccone *et al.*, 2009), delayed repair of DSB (Iwasa *et al.*, 2008). The molecular basis of these responses has not been clearly elucidated, but initial evidence suggested a potential role of survivin in controlling the expression of the DNA repair protein, Ku70 (Jiang *et al.*, 2009).

The data presented here extend these observations, and anticipate a role of the surviving spliced variant, ΔEx-3 (Li, 2005, Sampath & Pelus, 2007) in sensing the presence of unrepaired DNA damage in tumor cells.

This is a novel role for a molecule whose subcellular localization and function(s) have remained largely controversial in the literature, with reports variously implicating survivin-ΔEx-3 in nucleolar-dependent apoptosis inhibition (Song & Wu, 2005), mitochondrial control of Bcl-2 and caspase 3 activation (Wang *et al.*, 2002, Malcles *et al.*, 2007), or endothelial cell migration and invasion during angiogenesis (Caldas *et al.*, 2007). Here, survivin-ΔEx3 was characterized as an exclusively nuclear protein in tumor cells, whose stability in vivo and function in a DNA damage response were critically regulated by Chk2 phosphorylation (Reinhardt & Yaffe, 2009). This is consistent with an important role of post-translational modifications in controlling subcellular localization, protein-protein recognitions and modulation of apoptosis (Dohi *et al.*, 2007), and cell division (Vong *et al.*, 2005), of the survivin pathway.

Compelling evidence underscores a key role of Chk2 in a genomic integrity checkpoint after DNA damage. Activated via phosphorylation on Thr68 by the DNA damage sensor kinase, ataxia telangiectasia mutated (ATM), active Chk2 in turn phosphorylates a panoply of downstream targets implicated in checkpoint activation, cell cycle arrest, and apoptosis. In the

case of survivin-ΔEx3, loss of Chk2 phosphorylation nearly completely suppressed γH2AX reactivity, a marker of unrepaired DNA damage (Bonner *et al.*, 2008) important to recruit repair proteins to DNA foci (Paull *et al.*, 2000). The mechanistic requirements of this pathway have not been

completely elucidated, but data presented here indicate that Chk2-survivin-ΔEx3 signaling is specific for DSB, is independent of an MRN complex (Lavin, 2007), and does not involve homologous-directed DNA repair.

It is also clear that survivin-ΔEx3 does not directly regulate Chk2, as the overall levels of the kinase and its kinetics of activation in response to DNA damage were unaffected in the presence of phosphorylation-competent or –defective surviving-ΔEx3. Taken together, these findings suggest that Chk2 phosphorylation of survivin-□Ex3 may enhance sensing of DSB in tumor cells, i.e. via optimal γH2AX expression (Rodel *et al.*, 2011). This pathway may be temporally important at the earliest stages after DNA damage, in light of the rapid turnover of Chk2 phosphorylated survivin-ΔEx3, and, similarly to a proposed role of survivin, may result in rapid recruitment of repair proteins to DSB foci, including DNA-PK (Reichert *et al.*, 2011), or Ku70 (Jiang *et al.*, 2009). Functionally, this response would be expected to confer a survival advantage for tumor cells exposed to genotoxic stimuli. Consistent with this hypothesis, genome-wide bioinformatics studies presented here linked the expression of survivin-ΔEx3 to tumor progression, in vivo, in agreement with retrospective studies of patient cohorts (Koike *et al*., 2008, Nakano *et al.*, 2008), whereas, conversely, loss of Chk2 phosphorylation of survivin-ΔEx3 impaired long-term clonogenic tumor cell survival after DSB.

Aside from its tumor suppressive properties, which are inactivated by mutagenesis in a fraction of selected tumors (Motoyama & Naka, 2004), there has been considerable interest in exploiting the checkpoint function(s) of Chk2 for novel cancer therapeutics (Bolderson *et al.*, 2009). Under the

definition of “checkpoint abrogation”, this strategy relies on small molecule antagonists of Chk2 (as well as Chk1) to prevent cell cycle arrest in response to DNA damage, thus triggering apoptosis, mainly by mitotic catastrophe (Jiang *et al.*, 2009). However, the data presented here suggest that Chk2 may play a more direct role in tumor progression (El Ghamrasni *et al.*, 2011), potentially by exploiting multiple facets of the surviving pathway in transformed cells. In this context, the presence of activated Chk2 at the earliest stages of cellular transformation, in vivo (Gorgoulis *et al.*, 2005, Bartkova *et al.*, 2005), as exemplified here in the colorectal adenomato-carcinoma transition, has been interpreted as a barrier to tumor development (Gorgoulis *et al.*, 2005, Bartkova *et al.*, 2005), but may also paradoxically promote the acquisition of pivotal cancer traits, including increased resistance of apoptosis via the release of survivin from its mitochondrial stores (Ghosh *et al.*, 2008), and enhanced sensing of DNA damage via phosphorylation of survivin-ΔEx3 (this work).

In summary, we have identified a novel link between Chk2 and an alternatively spliced survivin variant, ΔEx3 in sensing DNA damage. In addition to further establishing the development of Chk2 inhibitors as a rational anticancer approach (El Ghamrasni *et al.*, 2011), these data reinforce the general contribution of survivin family proteins to multiple aspects of tumorigenesis (Altieri, 2010), and validate therapeutic strategies aimed at globally disabling aberrant transcription of the surviving gene during tumor progression (Iwasa *et al.*, 2008).

Chapter 6

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Appendix

ABBREVIATIONS

ABBREVIATIONS

This appendix contains a key of the most common acronyms and abbreviations used in this thesis.

ATCC = American Type Culture Collection
ATM = Ataxia-telangiectasia Mutated
ATR = Ataxia-telangiectasia and Rad3 Related
BER = Base Excision Repair
BIR = Baculoviral IAP Repeat
BSA = Bovine Serum Albumin
BRCA = Breast Cancer
BTCC = Bladder Transitional Cell Carcinoma
CARD = Caspase Recruitment Domain
CDK = Cyclin-Dependent Kinase
CHK1= Checkpoint Kinase 1
CHK2= Checkpoint Kinase 2
CHX = Cycloheximide
DED = Death Effector Domain
DMSO = Dimethyl Sulfoxide
DSB = Double Strand Break
EPR-1 = Effector Cell Protease Receptor-1
EDTA = Ethylenediaminetetraacetic Acid
Fas = Fibroblast-Associated protein
FBS = Fetal Bovine Serum
Flip = FADD-like interleukin-1-converting enzyme-inhibitory protein
GG-NER= Global Genomic Nucleotide Excision Repair
Hsp60 = Heat Shock Protein 60
Hsp90 = Heat Shock Protein 90
HR = Homologous Recombination
IAP = Inhibitor of Apoptosis Proteins
LB = Loading Buffer
LB medium =Luria-Bertani medium
LRR = C-terminal Leucine-rich Repeat

MMR = Mismatch Repair
MRI = Magnetic Resonance Imaging
MSI = Microsatellite Instability
NAIP = Neuronal Apoptosis Inhibitory Protein
NER = Nucleotide Excision Repair
NHEJ = Nonhomologous End-Joining
NOD = Nucleotide-binding Oligomerization Domain
ORF = Open reading frame
PBS = Phosphate-Buffered Saline
PCR = Polymerase Chain Reaction
PVDF = Polyvinylidene Fluoride
ROS = Reactive Oxygen Species
RPA = Replication Protein A
SDS = Sodium Dodecyl Sulfate
siRNA = Small Interfering Ribonucleic Acid
SSB = Single Strand Break
TAE = TRIS-Acetate-EDTA
TBE = Tris-Borate-EDTA
TGS buffer = Tris-Glycine-SDS buffer
TRAIL-R = TNF-Related Apoptosis-Inducing Ligand Receptor