The role of the inhibitor of apoptosis protein Survivin in cellular radiation response

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Summary

Survivin, the smallest and functionally unique member of the inhibitor of apoptosis protein (IAP) family, is frequently overexpressed in malignant cells and has been acknowledged as a predictive molecular marker for metastases and cancer patient survival following radiation therapy. The role of this protein in cellular radiation response, however, far exceeds a simple inhibition of apoptotic cell death involving non-caspase dependent mechanisms such as regulation of cell cycle and DNA damage repair. To investigate in more detail the role of Survivin in cellular radiation response and tumour cell motility, the present study aimed to establish and stably express several Survivin enhanced green fluorescent protein (EGFP)-tagged deletion or phosphorylation mutant constructs in SW480 colorectal cancer cells. To this end, Survivin wild-type (Surv. wt) and recombinant proteins lacking the binding sites for X-linked IAP (XIAP), Microtubules and heat shock protein 90 (Hsp90) (Survivin ΔXIAP/ΔMicTub/ΔHsp90) or the baculovirus IAP repeat (BIR) domain (Survivin ΔBIR) as well as phosphorylation mutants for the sites S20, T34 and T117 (Survivin S20A/S20D/T34A/T34D/T117A/T117D) were expressed in a pEGFP-N1 vector system. Subsequently, these mutant clones were subjected to more physiologic three-dimensional (3D) colony forming assays, immunofluorescence staining of DNA double strand break markers γH2AX and 53BP1, analysis of cell cycle distribution, induction of apoptosis, caspase 3/7 activity and transmigration assays following irradiation with doses ranging from 1 to 6 Gy. While knockdown of endogenous Survivin by RNA interference (siRNA) resulted in a significantly decreased 3D radiation survival in line with elevated numbers of residual γH2AX/53BP1 foci in pEGFP-N1 vector control, Survivin ΔXIAP, ΔBIR and T34A clones, both radiation clonogenic survival and the capacity to regulate DNA repair was rescued in clones stably overexpressing Survivin wt, ΔMicTub, ΔHsp90 and Survivin T34D mutant cells. Moreover, deletion of the BIR domain, XIAP, Microtubules and Hsp90 binding sites resulted in a G2/M cell cycle arrest, an elevated percentage of apoptotic SubG1 cells, an increased caspase 3/7 activity and significantly hampered transmigration activity of SW480 cells following depletion of endogenous Survivin. On a molecular level, constructs derived from Survivin ΔXIAP and T34A phospho-mutant overexpressing clones did not co-immunoprecipitate with the DNA repair protein DNA-dependent protein kinase catalytic subunit (DNA-PKcs), whereas Survivin wt and T34D constructs co-precipitated with the protein. These data confirm Survivin to act as a radiation resistance factor modulating cellular radiation response by multiple mechanisms including DNA DSB repair, induction of cell cycle arrest and apoptosis. It is for the first time indicated that Survivin's XIAP binding and T34 phosphorylation sites, but not its Microtubules and Hsp90 binding sites, are essential for radiation clonogenic survival and modulation of DNA damage repair, at least in

part by disturbing protein interaction with DNA-PKcs. On the contrary, XIAP, Hsp90 and Microtubules binding sites as well as the BIR domain were shown to be essential for proper cell cycle regulation, apoptosis inhibition and transmigration capacity of SW480 colorectal adenocarcinomas.

Zusammenfassung (summary in German)

Survivin, das kleinste und funktionell einzigartige Mitglied der *Inhibitor of Apoptosis Protein* (IAP) Familie ist in der Mehrzahl maligner Zellen überexpremiert und stellt einen wesentlichen prädiktiven molekularen Marker für eine Metastasierung und das Überleben von Patienten nach Strahlentherapie dar. In diesem Zusammenhang wurde zudem gezeigt, dass die Rolle von Survivin in der zellulären Strahlenantwort weit über eine alleinige Hemmung der apoptotischen Reaktionskaskaden hinausgeht, sondern vielmehr auch Caspase-unabhängige Mechanismen wie eine Regulation des Zellzyklus und der Reparatur von DNA-Schäden beinhaltet. Um die Rolle von Survivin in der zellulären Strahlenantwort und Tumorzellmotilität detaillierter zu untersuchen, war es Ziel der vorliegenden Arbeit, eine Reihe von grün fluoreszierendes Protein (GFP) markierten Survivin Deletions- und Phosphorylierungsmutanten zu generieren und stabil exprimierende Klone kolorektaler SW480 Karzinomzellen herzustellen. Dazu wurden wildtyp-Survivin und rekombinante Proteine mit fehlenden Bindungsstellen für X-linked IAP (XIAP), Mikrotubuli, Hitzeschockprotein 90 (Surv-ΔXIAP/ΔMicTub/ΔHsp90), die *Baculovirus IAP Repeat* (BIR) Domäne (Surv-ΔBIR), sowie Mutanten der Phosphorylierungsstellen S20, T34 und T117 (Survivin S20A/ S20D/T34A/T34D/T117A/ T117D) in einem pEGFP-N1 Vektor System zur Expression gebracht. Anschließend wurden die Zellen unter physiologischen Bedingungen in drei-dimensionalen (3D) Koloniebildungstests eingesetzt, die Expression der DNA-Doppelstrangbruchmarker γH2AX und 53BP1 mittels Immunfluoreszenz quantifiziert und die Zellzyklusverteilung, Induktion von Apoptose und die Caspase 3/7 Aktivität bzw. die Transmigrationsfähigkeit nach Bestrahlung mit Dosen von 1 bis 6 Gy untersucht. Während die Hemmung von endogenem Survivin durch RNA-Interferenz (siRNA) zu einer signifikanten Minderung des 3D klonogenen Überlebens und einer erhöhten Anzahl residueller γH2AX/53BP1 Foci in pEGFP-N1 Kontrollvektor, Surv. ΔXIAP, Surv. ΔBIR und Surv. T34A transfizierten Zellen führte, konnte das radiogene Zellüberleben und die DNA-Reparaturkapazität in stabil Surv. wt, Surv. ΔMicTub, Surv. ΔHsp90 und Surv. T34D überexprimierenden Zellklonen rekonstituiert werden. Darüber hinaus führte die Deletion der BIR Domäne, der Mikrotubuli und der Hsp90 Bindungsstelle zur Induktion eines G2/M Zellzyklus-Arrestes, einem erhöhten Anteil apoptotischer SubG1 Zellen, einer gesteigerten Caspase 3/7 Aktivität und einer signifikanten Hemmung der Transmigration von SW480 Zellen nach Attenuation des endogenen Survivins. Auf molekularer Ebene gelang bei Konstrukten aus Surv. ΔXIAP and T34A Phospho-mutanten überexpremierenden Zellen keine Ko-Immunpräzipitation mit dem DNA Reparaturprotein DNA Proteinkinase (DNA-PKcs), während dies bei wt-Survivin Klonen sowie bei der T34D phospho-mimetischen Mutante möglich war. Zusammengefaßt bestätigen diese Daten die Aktivität von Survivin als

Radioresistenzfaktor, der die zelluläre Bestrahlungsantwort durch multiple Mechanismen, einschließlich der DNA- Doppelstrangbruch-Reparatur und der Induktion eines Zellzyklus Arrestes und von Apoptose zu modulieren vermag. Darüber hinaus konnte hier erstmals gezeigt werden, dass die XIAP Bindungs- und T34 Phosphorylierungs-, nicht jedoch die Microtubuli und Hsp90 Bindungsstellen essentiell für das klonogene Zellüberleben nach Bestrahlung und die Modulation der DNA Schadensreparatur sind. Dies beruht, zumindest teilweise, auf der Beeinträchtigung der Interaktion mit dem Protein DNA-PKcs. Im Gegensatz dazu sind für die Regulation des Zellzklus, die Inhibition der Apoptose und das Migrationsverhalten der kolorektalen Adenokarzinomlinie SW480 sowohl die XIAP-, die Hsp90- und die Mikrotubuli-Bindungsstellen wie auch die BIR-Domäne wesentlich.

1. Introduction

1.1 Colorectal cancer

Colorectal cancer (CRC), the second most common cancer in women and third in men, accounts for 1,233,700 cancer incidents annually worldwide, 608,700 cases of which eventually lead to death [1]. More than 95% of CRC cases are adenocarcinomas while 50% of the patients are diagnosed with distant metastasis or local recurrence with a median survival time of 4 to 22 months [2]. Although there are some environmental and lifestyle risk factors it is nowadays generally accepted that colorectal cancer is caused by changes in various molecular pathogenic pathways, such as CpG island methylator phenotype (CIMP), chromosomal instability (CIN) or microsatellite instability (MSI) [3].

The CIN pathway is the one via which approximately 85% of CRCs are developed [4] mainly due to an accumulation of numerical or structural chromosomal anomalies [5]. In this pathway the earliest detectable lesion is a microscopic mucosal dysplasia named aberrant crypt focus (ACF) [6] which is followed by the development of a polyp [3, 7]. Typical aberrations during the CIN pathway include V-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue (KRAS) oncogene mutation, deletion of 17p chromosome (which entails the tumour suppressor gene TP53) and loss of 18q chromosome [3, 4]. However, there are many alternative genetic and epigenetic changes that have recently been described [8] and will eventually be considered as complementary events to the aforementioned genetic mutations [3].

A deficiency in the function of the tumour suppressor TP53 gene, typically via allelic loss of 17p [3], is known to characterize 50-75% of CRCs [9]. The TP53 protein is responsible for the slowdown of cell cycle in order for the DNA damage to be repaired [3] as well as for the

induction of pro-apoptotic genes in case of an accumulation of unrepaired genetic damage [10].

Around 60% of CRCs demonstrate an allelic loss of 18p21.1, a site entailing SMAD2 and SMAD4 [11], both of them being involved in the transforming growth factor-β (TGF-β) signaling pathway which is essential for apoptosis and growth regulation [3].

In the case of MSI, microsatellites typically consist of 1-5 nucleotide tandem repeats found throughout the whole genome and MSI corresponds to an inconsistency in the amount of those microsatellites between normal and tumour cells [3, 12]. MSI phenotype can arise from mutations interfering with the mismatch repair (MMR) machinery, a condition present in approximately 15% of colorectal cancers [13-15]. It is now understood that the colorectal cancer genome entails probably thousands molecular aberrations certain subsets of which take part in carcinogenesis [12].

Approximately 15% of all sporadic colorectal cancers are originated by the CIMP pathway [16, 17]. KRAS, v-Raf murine sarcoma viral oncogene homologue B1 (BRAF) and MSI mutations often occur in the case of CIMP cancers which are also related to older age, genetic predisposition or the female gender [18]. In general, genetic and epigenetic aberrations can take place in numerous combination patterns with some genes exclusively modified by epigenetic inactivation and others being both genetically and epigenetically inactivated [18].

Coloretal cancer therapeutic approaches vary depending on numerous factors such as tumour localization and stage, involvement of tumour positive lymph nodes etc., with the majority of such therapies to involve surgical resection followed by adjuvant chemotherapy in case of colon carcinoma. By contrast, neoadjuvant (preoperative) combined radiation and chemotherapy (comprised of 5-Fluorouracil along with additional agents such as oxaliplatin, or irinotecan) became standard therapy for patients with locally advanced rectal cancer [19].

1.2 Inhibitor of apoptosis proteins (IAPs)

In 1993 a baculovirus gene that inhibits apoptosis in virally infected *Spodoptera frugiperda* insect cells was discovered [20] and since then several cellular homologues have been detected in yeast, nematodes, flies and higher vertebrates. It is generally known that IAPs are important for cell division, morphogenesis, nuclear factor kappa B (NF-κB) activation, heavy metal homeostasis and Mitogen-activated protein (MAP) kinase signaling [21]. IAPs are characterized by the presence of a variable number of Baculoviral IAP Repeat (BIR) motifs comprised of approximately 70 amino acids coordinating a zinc ion through cysteine and histidine residues [21]. Apoptosis being a controlled cell suicide process which plays a pivotal role in homeostasis and development of the organism, when deregulated can lead to various pathogenic conditions including cancers, neurological disorders and autoimmunity [21]. The fact that many neoplasms are found to express a high level of IAPs along with a genetic translocation regarding the gene encoding cellular IAP2 (c-IAP2) detected in a subset of B cell lymphomas led to the notion that IAPs contribute to apoptotic cell death resistance that characterizes numerous cancers [21]. It was originally thought that those proteins were physical caspase inhibitors representing a cytoprotective factor downstream of death receptors [22, 23]. It is now obvious that IAPs not only regulate caspases via mechanisms distinctive for each member of the family [24] but also participate in several aspects of cellular homeostasis [21, 25].

The function of IAPs depends not only on their distinct domains but also on posttranslational modifications and changes at the level of expression [21]. In particular, protein-protein interactions, intracellular localization and IAP stability are influenced by phosphorylation of some IAPs [26-28] while interactions among IAP molecules can have an effect on their expression levels [29-32].

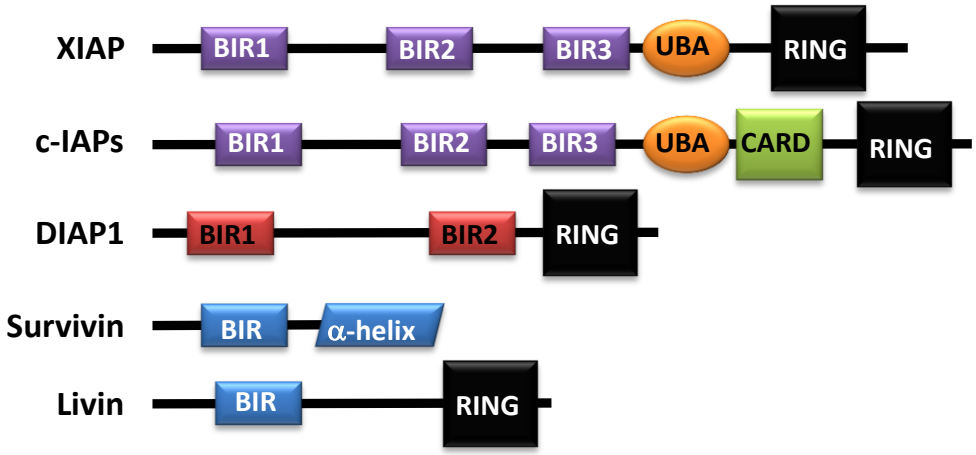


Figure 1: Symbolic representation of structural domains of IAPs. Domains such as the BIR (Baculovirus IAP repeat) and RING (really interesting new gene) shown here characterize all members of the IAP family (except Survivin). DIAP1: Drosophila IAP 1; UBA: binding site for polyubiquitinated proteins; CARD: caspase-associated recruitment domain; c-IAPs: cellular IAPs; XIAP: X-linked inhibitor of apoptosis protein. (Figure adapted from [22])

A structural characteristic common to all IAPs is the BIR domain which can be found either in a single copy or two to three repeats in the N-terminus along with other functional domains like a really interesting new gene (RING) or caspase-associated recruitment domain (CARD) in the proximity of C-terminal region of the proteins [21] (Figure 1). BIR domains fold as three-stranded β-sheets surrounded by four α-helices and their signature sequence is $CX_2CX_{16}HX_6C$, where C = cysteine, H = histidine and X stands for any amino acid [33-36]. C-IAP1 (cellular IAP1), c-IAP2 (cellular IAP2) and XIAP (X-linked IAP) contain three BIRs and one RING domain, functioning as an E3 ubiquitin ligase (a ubiquitin-associated domain involved in binding to ubiquitinated proteins) and a CARD in c-IAP1 and c-IAP2 whose function is not fully understood [22]. In spite of the resemblance all BIR domains bear, different BIRs identify different intermediates and participate in distinct signaling pathways [21].

In principal, BIRs are involved in protein-protein interactions as well as protein recognition [21], as a peptide-binding groove (in the case of XIAP, c-IAP1 and c-IAP2) comprises a hydrophobic recognition site for IAP-binding motif (IBM) entailing proteins [22]. Certain

apoptosis inducers such as second mitochondrial-derived activator of caspase (Smac) also known as direct IAP-binding protein with low isoelectric point (DIABLO) along with initiator capsase 9 and effector caspase 7 contain an IBM i.e. a tetrapeptide region with an invariant N-terminal alanine residue and other conserved residues [37, 38]. Short peptides that contain SMAC IBM can prevent BIR3 from binding to caspase 9 [39]. The binding of XIAP and Smac is greatly increased due to the tandem presence of BIR2 and BIR3 in XIAP, as Smac forms a stable complex with XIAP in a ratio of 2:1 via concurrent interaction with both BIR2 and -3 [21]. Not every BIR domain contains an IBM recognition site [40] as for example, the XIAP BIR1 does not bind caspases or IBM containing proteins, but identifies molecules involved in NF-κB activation instead [41, 42].

In general, the interaction between caspases and IAPs occurs via IBM-dependent complexes, resulting in the abolishment of enzymatic activity of caspases [22, 38]. While this was originally thought to be the sole role of IAPs [43], it is now commonly accepted that only XIAP (from the mammalian IAPs) actually inhibits caspases *in vivo* [25]. XIAP interacts with caspases 3, 7 and 9, obliterating their cell killing ability [22]. The XIAP linker region upstream of BIR2 interacts with the catalytic cleft of caspases 3 and 7, inhibiting substrate accessibility thus eliminating their activity [44-46]. Caspase 9 is inhibited via XIAP BIR3 as the latter interferes with the homodimerization domain of the enzyme, abolishing the conformational change required for activity [47]. XIAP participates also in cell-death regulation through its RING domain [21] the mechanism of which has not yet been clarified, although recent studies revealed a cytoprotective role for the RING domain when mice expressing a BIR-only type of XIAP showed an increased caspase activity and cell death *in vivo* [48].

Members of the IAP family bind to each other via their BIR domains [21]. For instance, the BIR1 and -3 from XIAP are responsible for binding with the single BIR of Survivin in order to form a complex of increased stability against proteasomal degradation and synergistically inhibit apoptosis [31]. Survivin BIR also binds Aurora B, a serine/threonine kinase involved in

the regulation of cell division, as well as Borealin and inner centromere protein (INCENP) thus forming the chromosomal passenger complex (CPC) [49].

The RING domain is characterized by the presence of one or two histidines and six to seven cysteines comprising a cross-brace structure that coordinates two zinc ions [50]. These domains mediate ubiquitin protein ligase (E3) activity as the transfer of ubiquitin to target proteins is facilitated by a ubiquitin activating enzyme (E1) and a ubiquitin conjugating enzyme (E2) [51]. All IAPs that entail RING domains demonstrate E3 activity involving apoptotic and signaling molecules as well as themselves in a homo- or heterotypic fashion [21].

In cells exposed to pro-apoptotic stimuli, XIAP, c-IAP1 and c-IAP2 undergo autoubiquitination in a RING dependent manner, a process found abrogated in a RING-mutant XIAP lacking E3 activity [52, 53]. Those pro-apoptotic stimuli are able to cause IAP autoubiquitination due to IBM-containing IAP antagonists that are released [54].

Apart from caspase inhibition, IAPs were also found to modulate NF-κB, a transcription factor pivotal for inflammation, cell survival, migration, invasion and immunity [55-57]. Consequently, IAP-mediated NF-κB activation can result in tumour progression and metastasis [58].

IAPs are found to be overexpressed in many tumour entities as inhibition of apoptosis plays a major role in carcinogenesis by enabling cell survival in unfavourable conditions typically hostile to non-cancerous cells. For instance, the fact that Survivin is overexpressed in breast, colorectal, esophageal/gastric carcinoma, lymphoma and neuroblastoma as well as the poor prognosis with which this overexpression is associated with, led to the hypothesis that IAP up-regulation might comprise an oncogenic event [59, 60]. Besides Survivin (which will be further analyzed in section *1.4*), c-IAP1 and c-IAP2 also play a role in mammalian cancers with high levels of c-IAP1 to promote carcinogenesis [61, 62] and c-IAP2 being correlated

with a type of neoplasia called MALT (mucosa-associated lymphoid tissue) lymphomas [63]. Another way for tumour cells to evade apoptosis is to down-regulate molecules that cause suppression of XIAP's caspase-inhibitory activity, such as XIAP-associating factor 1 (XAF1) whose levels have been reported to be decreased in some cancer cell lines [64, 65].

As IAPs are implicated in tumourigenic events, their expression has been targeted via RNA interference methods and alternatively, compounds mimicking naturally occurring IAP antagonists have been developed in order to neutralize IAP function [66]. For instance, Survivin suppression has been shown to sensitize tumour cells to both chemo- [59, 67, 68] and radiation therapy.

1.3 DNA damage repair

Higher eukaryotic cells have in the course of evolution acquired complex molecular mechanisms to preserve chromosomal integrity in their genomes amid background radiation, byproducts of cellular metabolism and environmental mutagens [69]. Maintaining genomic stability is essential for prevention of chromosomal rearrangements that can result in cancer via altered gene expression [70, 71]. There is a wide range of different types of DNA damage that can arise within the cell, with the DNA double-strand breaks (DSBs) being of great importance as they can cause cell death or cancer if improperly repaired [69, 72]. Ionizing radiation, reactive oxygen species as well as certain types of anti-cancer drugs are able to give rise to this type of lesion which is met mainly by two different repair mechanisms that mammalian cells have evolved: homologous recombination (HR) and non-homologous end joining (NHEJ) [69]. An intact sister chromatid is required for HR to take place which is, therefore, restricted to the S and G2 cell cycle phases. On the other hand, in the case of NHEJ the two broken DNA ends are re-joined without the need of a sister chromatid, a fact

that renders NHEJ functional in all cell cycle phases and thus the major DSBs repair mechanism in mammalian cells.

A protein kinase central to the damage response signal transduction regarding DSBs is the ataxia telangiectasia mutated (ATM) [73] which is recruited to DSB lesions via the Mre11-Rad50-Nbs1 (MRN) complex [74]. Following ATM activation (after being auto-phosphorylated) [75] another part of this signalling cascade is the phosphorylation of a histone H2A variant, thus, generating γH2AX which in turn facilitates the recruitment of other key proteins [such as p53-binding protein 1 (53BP1), mediator of DNA damage checkpoint protein 1 (MDC1), breast cancer 1 early onset (BRCA1), etc.] at DSB sites [76, 77].

NHEJ is the predominant pathway through which irradiation-induced DSBs are repaired [78-81]. This process is known to commence with the binding of Ku70 and Ku80 proteins, to double-stranded DNA ends in a manner that allows Ku to translocate along the DNA [82-84] and eventually recruit a large catalytic subunit of the DNA-dependent protein kinase complex (DNA-PKcs) [85, 86]. DNA-PKcs seems to be responsible for the regulation of the DNA ends processing to generate the 3'-OH and 5'-P ends required for ligation [87, 88]. The assembly of Ku and DNA-PKcs on DNA ends is followed by recruitment of a complex termed DNA ligase IV-X-ray repair cross-complementing protein 4 (XRCC4) that facilitates the re-joining step [89-92].

1.4 Survivin

Survivin, a 16.5 kDa protein of 142 amino acid residues encoded by the *BIRC5* gene, is a member of the IAP family firstly described by *Ambrosini et al.* in 1997 [93]. Survivin is the smallest member of this protein family consisting of a single BIR domain [36] and an extended C-terminal α-helix [93]. When in solution, Survivin forms a stable homodimer [36] while clear evidence rendering such an architecture necessary for function is still missing. On

the other hand, it is the monomeric protein that is involved in nuclear-cytoplasmic shuttling [94] and key protein recognition such as binding to the chromosomal passenger protein Borealin [49, 95].

Survivin has been shown to function in a cytoprotective manner when in cytoplasm due to its anti-apoptotic activities [96] while when located in the nucleus it participates in cell division [97] and DNA repair regulation [98, 99]. It is expressed in a cell-cycle regulated manner with a peak at mitosis [97] showing a 40-fold up-regulation in G2/M phase when found to be localized in the mitotic apparatus [100]. Thus, Survivin is acknowledged to be an indispensable member of the chromosomal passenger complex [101, 102].

Homozygous deletion of Survivin gene resulted in early embryonic death [103], while mitotic defects, cell death and tissue involution followed after conditional deletion of Survivin in adult tissues [104, 105]. Such a scenario has been repeatedly confirmed by studies conducted on Survivin orthologues in other model systems like *C. elegans* [106, 107] and yeast [108], demonstrating pivotal roles of this protein in mitosis regarding chromosomal segregation and cytokinesis. Survivin physically interacting with other members of the chromosomal passenger complex such as Borealin, Aurora B and INCENP [49], regulates chromosomal alignment, cytokinesis as well as chromatin-associated spindle assembly [102], whereas another pool of this protein stabilizes the mitotic spindle [109]. In order to do this, Survivin binds to polymerized microtubules through its C-terminal α-helices [22] suppressing microtubule dynamics [110]. Those different pools of Survivin manage to work together probably due to post-translational modifications such as monoubiquitination by Lysine 48 (L48) and L63, an event necessary for mitotic trafficking [111].

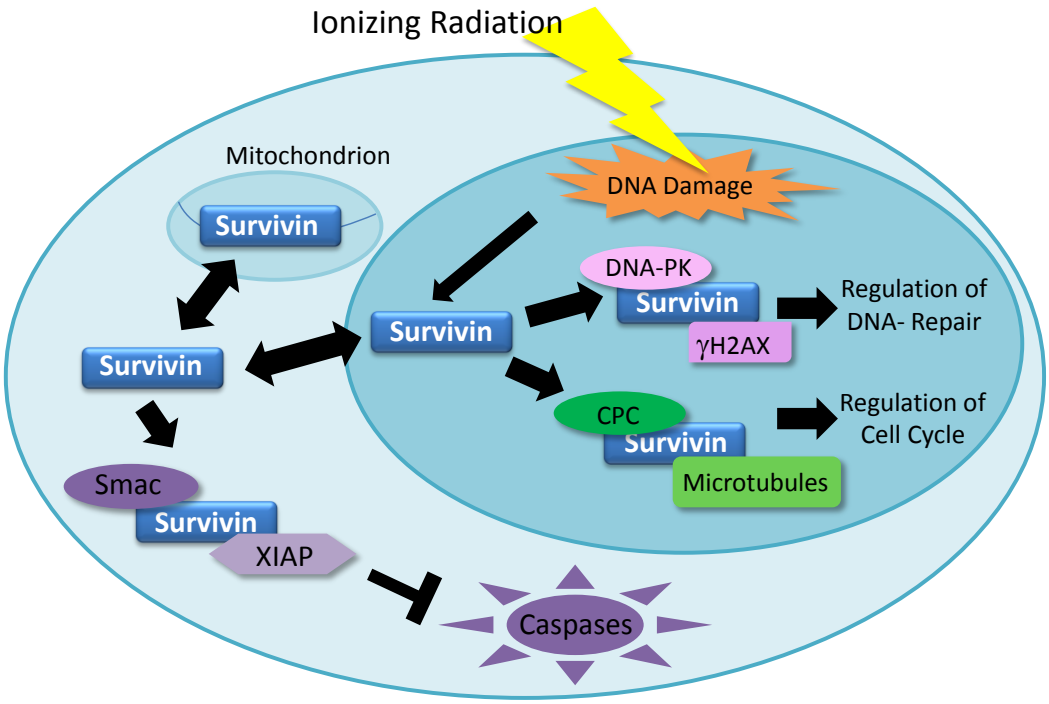


Figure 2: Schematic diagram of Survivin role in radiation response. When in the cytoplasm Survivin interacts with other members of the IAP family (such as XIAP) to inhibit apoptosis while when found in the nucleus it participates in cell division (being a member of the CPC) as well as it regulates DNA repair (interacting with DNA-PKcs and other members of the DNA repair machinery). XIAP: X-linked IAP; CPC: Chromosomal passenger complex; DNA-PKcs: DNA-dependent protein kinase catalytic subunit; Smac: Second mitochondria-derived activator of caspases; (Figure adapted from [112])

Survivin being a multifunctional protein, interacts with a variety of protein partners in order to co-ordinate its different activities including apoptosis inhibition (via interaction with other IAPs), cell division (being a member of the CPC) and regulation of DNA DSBs repair (interacting with DNA-PKcs, Ku-70, 53BP1, etc.) (Figure 2) [98, 99, 112].

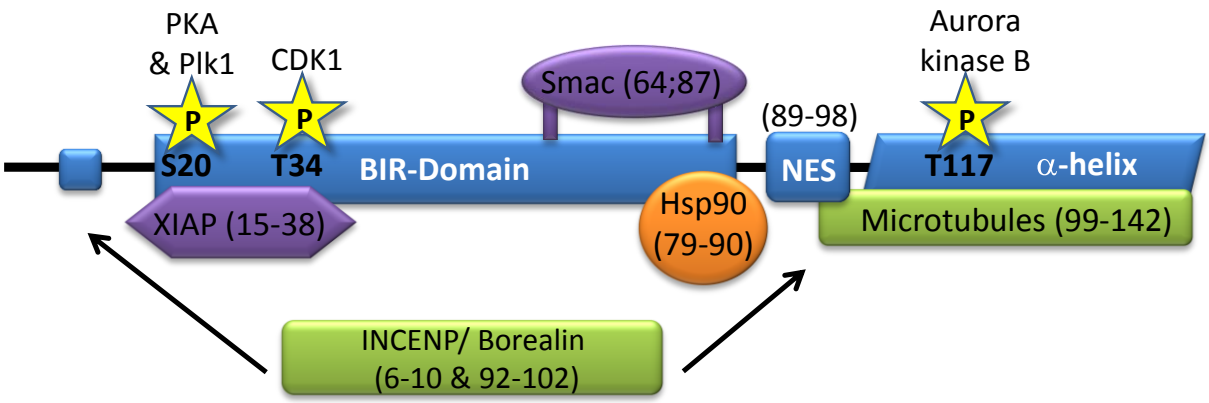


Figure 3: Symbolic illustration of Survivin protein structure. Functional domains, binding sites as well as phosphorylation sites of Survivin are shown here. XIAP: X-linked inhibitor of apoptosis protein; Smac: Second mitochondria-derived activator of caspase; INCENP: Inner centromere protein; Hsp90: Heat shock protein 90; NES: Nuclear export signal; PKA: Protein kinase A; Plk1: Polo-like kinase 1; CDK1: Cyclin-dependent kinase 1; S20: Serine 20; T34: Threonine 34; T117: Threonine 117. (Figure adapted from [112])

Survivin entails various phosphorylation sites such as Serine 20 (S20), Threonine 34 (T34) and Threonine 117 (T117) [113-116] via which both protein stability and trafficking among the subcellular compartments are achieved [112]. There are also several molecules that interact with this protein including tubulin, heat shock protein 90 (Hsp90) [117], Hsp60 [118], aryl hydrocarbon receptor-interacting protein (AIP) [119] and other members of the IAP family like XIAP [58] (Figure 3).

Except for XIAP, all other IAPs including Survivin do not show a direct binding to caspases [21]. In the case of Survivin, it is believed that apoptosis is inhibited only after its complex-formation with XIAP [31] where Survivin BIR residues 15-38 [114] interact with BIR1 and BIR3 of XIAP [31]. Besides caspase inhibition, the Survivin-XIAP complex promotes XIAP stability (as well as Survivin stability) against ubiquitin-dependent degradation and it also leads to tumour growth *in vivo* [114] being involved in XIAP-mediated NF-κB activation [58]. The latter event gives rise to NF-κB-dependent transcription of fibronectin (an extracellular matrix protein) [58] which in turn engages β1 integrins at the cell surface activating cell

motility kinases, proto-oncogene c-Sarcoma (Src) and focal adhesion kinase (FAK) resulting in enhanced tumour cell migration, invasion and metastatic dissemination *in vivo* [58]. In order to regulate other functions (besides apoptosis inhibition) such as cytokinesis or mitotic spindle checkpoint, Survivin co-operates with other IAPs like BRUCE and c-IAP1 [28, 31, 120].

A plethora of pre-clinical studies on various cancer cell lines have shown that down-regulation or antagonization of Survivin led to inhibition of tumour cell proliferation along with increased apoptosis as well as greater sensitivity to TNF-related apoptosis-inducing ligand (TRAIL), tumour necrosis factor-α (TNF-α) and chemotherapeutic drugs in both cell culture and xenograft models [121, 122]. It is further known that Survivin renders a radiation resistance factor [123-125] the attenuation of which exerts a radiosensitization effect on tumour cells. For instance, Survivin down-regulation combined with ionizing radiation was followed by a diminished clonogenic survival *in vitro* (regarding colorectal carcinoma, glioblastoma, hepatocellular carcinoma and NSCLC cells) as well as tumour growth retardation in xenograft models [112]. The mechanisms behind such observations are indeed complex, by far exceeding a mere irradiation-induced apoptotic cell death [112]. In particular, inability to repair DNA damage is one of the caspase-independent mechanisms via which Survivin down-regulation can decrease tumour cell survival after irradiation, firstly described by *Chakravarti et al.* [124].

Following this, Survivin-specific siRNA, anti-sense oligonucleuotide (ASO) or a small molecule inhibitor of Survivin expression called YM155, used in colorectal and non-small-cell lung carcinoma (NSCLC) cells showed a hampered DNA damage repair [126-128]. Additionally, Survivin was observed to accumulate in the nucleus after radiation exposure as well as directly interact with DNA DSB repair proteins such as DNA-PKcs, Ku70, MDC1, γH2AX and 53BP1 in colorectal cancer cells and glioblastoma cells [98, 99]. Apart from a diminished radiation clonogenic survival following Survivin attenuation, both DNA-PKcs

kinase activity and the level of S2056 autophosphorylation of DNA-PKcs were significantly decreased [98, 99].

In general, interference with IAP function or expression can seriously affect cellular homeostasis leading to human diseases, most importantly cancer. In neuroblastoma for instance, the Survivin locus on 17q25 has often been seen amplified [129] and in genome-wide studies it was found to be the fourth top 'transcriptome' in malignancies of colon, lung, breast, brain and melanoma while being low or undetectable in the corresponding normal tissues [130]. However, this 'cancer-specific' overexpression of Survivin has not yet been fathomed. Survivin gene expression is up-regulated probably through various oncogenic pathways while other mechanisms are responsible for the silencing of Survivin gene via several tumour suppressor networks [131]. In normal tissues for example, Survivin levels can be maintained low thanks to activated tumour suppressor mechanisms [132], whereas the oncogene activation that takes place in transformed cells and is associated with loss of tumour suppression may be responsible for induction of Survivin gene expression *in vivo* [131].

Some of the oncogenic pathways potentially responsible for the up-regulation of Survivin gene expression are the v-Ha-ras Harvey rat sarcoma viral oncogene homologue (c-Ha-Ras) [133], v-myc myelocytomatosis viral oncogene homologue (avian) (c-Myc) [134], wingless-related integration site (WNT)/β-catenin/transcription factor 4 (TCF4) [135], neurogenic locus notch homologue (Notch) [136] as well as transcription factors such as signal transduction and activator of transcription 3 (STAT 3) [137], E2F activators [138], NF-κB [139] and hypoxia inducible factor 1α (HIF-1α) [140].

On the other hand, for the transcriptional repression of Survivin non-mutated tumour suppressor TP53, wild type adenomatous polyposis coli gene (APC) [141] and phosphatase and tensin homologue deleted from chromosome ten (PTEN) [142] are among the

candidates. 82% of rectal and 60% of colonic cancers are characterized by APC mutation [143] which appears to trigger colorectal carcinogenesis as this mutation results in up-regulation of Survivin transcription via activation of β-catenin/TCF-4 signaling [144]. The mechanisms however, through which Survivin transcription is eventually repressed or up-regulated are not yet clear although it is assumed that they include direct binding to the Survivin promoter or modification of chromatin structure accessibility within the promoter region in the case of TP53 [145, 146] or attachment of forkhead box O1 (FOXO1) and FOXO3a transcription factors [142] (or APC protein in the case of PTEN [147]) onto the Survivin promoter. Another factor that is known to bind the Survivin promoter repressing transcription via epigenetic chromatin modifications, is the histone deacetylase silent mating type information regulation 2 homologue 1 (SIRT1) which is *de novo* transcribed via BRCA1 [148, 149].

In addition, non-transcriptional mechanisms may also interfere with Survivin expression in tumours, such as stabilization of Survivin mRNA in an mammalian target of rapamycin (mTOR)-mediated pathway described in prostate cancer [150].

According to numerous retrospective analyses of series of patients and genome-wide microarray studies, Survivin comprises a therapy resistance factor contributing to overall dismal disease outcome [59]. In particular, deregulated Survivin has an impact on mitotic transitions amongst tumour cells thus enabling viability of aneuploid cells [151], overcoming cell-cycle checkpoints [152], conferring resistance to microtubule-targeting molecules [153], as well as synergistically interacting with oncogenes such as Myc [154].

At the moment, several phase I and II clinical trials targeting Survivin are either in progress or already completed, with a focus on the application of a second generation 2'-O-methoxy-methyl modified ASO (LY2181308 or *Gataparsen*, Eli Lilly and Company, Indianapolis, USA),

the transcriptional inhibitor YM155 (Astellas Pharma Inc., Tokyo, Japan) as well as immunotherapeutic approaches [148].

Patients diagnosed with breast/colon cancer, lymphoma, leukemia or melanoma have demonstrated spontaneous anti-Survivin T-cell reactivity [155] providing compelling evidence that Survivin could be recognized as a 'non-self' protein in cancer patients mounting an immune response against it [156]. Thus, Survivin renders a potential vaccination target against cancer [157], as autologous cytotoxic T lymphocytes (Survivin directed) activated with Survivin-primed dendritic cells or Survivin peptides are now being used in phase I trials in recurrent oral/colorectal cancer [158, 159], myeloma [160] and malignant melanoma [161].

Apart from a feasible therapeutic target, Survivin renders a diagnostically relevant prognostic marker [148] associated with more aggressive clinicopathologic features along with a higher likelihood of tumour recurrence and reduced disease-free survival rates [59, 148, 162].

1.5 Aim of project

The aim of this project was to further examine the role of Survivin in radiation survival of tumour cells with respect to clonogenic survival, apoptosis, cell cycle regulation, cell motility and DNA damage response. The course of action involved the development of specific Survivin deletion and phosphorylation mutants followed by their functional characterization.

The deletion mutants created were the ΔXIAP, ΔMicTub, ΔHsp90 and ΔBIR lacking the XIAP, Microtubules, Hsp90 binding sites and BIR domain of the protein, respectively (Figure 3), whereas the phosphorylation sites S20, T34 and T117 of Survivin were mutated to Alanine (kinase-dead) S20A, T34A, T117A or to Aspartic acid S20D, T34D and T117D (phospho-mimetics). The mutants were subsequently inserted into a C-terminal expression vector, namely the pEGFP (enhanced green fluorescent protein). The EGFP- tagged deletion as well

as phosphorylation constructs along with the corresponding Survivin-EGFP wild type (wt) and empty vector (pEGFP) control were then used to stably transfect the human colorectal cancer cell line SW480. The cells stably expressing the Survivin-EGFP fusion constructs were then subjected to various functional assays including more physiological three-dimensional (3D) clonogenic radiation survival assays, conventional 2D and more physiological 3D immunofluorescence staining of γH2AX/53BP1 foci used as markers for DNA DSBs, invasion/transmigration assays, cell cycle analysis (flow cytometry), apoptosis (caspase 3/7) assays as well as co-immunoprecipitation assays following subcellular fractionations.

2. Materials and Methods

2.1 Materials

2.1.1 Appliances/Instruments

Appliance	Model/Description	Company
Agarose- Gel electrophoresis- chamber		peQLab Biotechnologie GmbH, Erlangen
Linear Accelerators	SL-15	Elekta, Crawley, UK
Electrophoresis chamber, Glass plates, Combs		Bio-Rad Laboratories, Munich
ELISA Reader	VIKTOR™ 1420 Multilabel Counter Software: Wallac 1420n Manager	Wallac, Waltham, MA, USA
Developer	Optimax Typ TR	HS Laborgeräte, Wiesloch
Flow Cytometer	FACSCalibur Software: Cell Quest Pro	Becton DICKINSON, Heidelberg
Water bath		Gesellschaft für Labortechnik, GmbH, Burgwedel
IKA® Shaker MTS 4		IKA Labortechnik, Staufen
Incubator	HERA cell 240+240i	Thermo Fisher Scientific, Dreieich
Microscope	AxioImager Z1 Axio Vision Imager Softwear 4.6	Zeiss, Jena
pH Meter	pH Meter 765 Calimatic	Knick, Berlin
Photometer	Bio Photometer	Eppendorf, Hamburg
Shaker-Incubator	ES-20	BioSan, Riga, Lettland
Laminar flow hood	HERA safe	Thermo Fisher Scientific,

		Dreieich
Thermocycler	Primus 96 advanced	PEQLab Biotechnologie GmbH, Erlangen
Semi-Dry Transfer Cell	Transblot SD	Bio-Rad Laboratories, München
Centrifuges	mini Spin	Eppendorf, Hamburg
	UNIVERSAL 320R	Hettich, Tüttlingen
	Eppendorf 5810	Eppendorf, Hamburg

Note: The *mini Spin* is a tabletop centrifuge (suitable for 1.5 ml and 2 ml microcentrifuge tubes only) with a rotor radius of 6 cm. The *UNIVERSAL 320R* is a benchtop centrifuge with a rotor radius of 85 mm for 1.5 ml and 2 ml microcentrifuge tubes and another rotor with a radius of 141 mm for 15ml and 50ml tubes. In this work, all the room temperature centrifugation steps of microcentrifuge tubes were performed in the *mini Spin* centrifuge, while all the 4°C centrifugation steps were performed in the *UNIVERSAL 320R*.

2.1.2 Consumables

Description	Company
8 Stripwell™ Plates, Flat Bottom, Certified High Binding	Corning Inc., NY, USA
96 Well micro-plates	Greiner Bio-One, Frickenhausen
100 mm cell culture dishes	Sarstedt AG & Co, Nümbrecht
60 mm cell culture dishes	Sarstedt AG & Co, Nümbrecht
Amersham™ Hyperfilm™ ECL High performance chemiluminescence film	GE Healthcare, BKM, UK
Blotting membranes GB003	VWR, Darmstadt
Amersham™-Hybond™-ECL	GE Healthcare, BKM, UK
Culture slides 8 chambers	BD Falcon™, Eremodegem, Belgium
CELLSTAR® 6-well cell culture plates	Greiner Bio-One, Frickenhausen
CELLSTAR® 12-well cell culture plates	Greiner Bio-One, Frickenhausen
CELLSTAR® 24-well cell culture plates	Greiner Bio-One, Frickenhausen

CELLSTAR® Filter Top cell culture flasks	Greiner Bio-One, Frickenhausen
Cloning Cylinders	Scienceware®, Pequannock, NJ, USA
Cover foil, Easy seal (80x140 mm)	Greiner Bio-One, Frickenhausen
Culture slides 8 Chambers	BD Falcon™, Erembodegem, Belgium
15ml tubes	Greiner Bio-One, Frickenhausen
50ml tubes	Greiner Bio-One, Frickenhausen
Filter paper	Whatman, Kent, UK
Fuji Medical X-Ray Film / Super RX	Fujifilm Holdings Corporation,Dusseldorf
Microscopic Slides	Thermo Fisher Scientific, Dreieich
Microscope Cover Glasses (24x60mm)	Marienfeld, Nordrhein-Westfalen
Microscope Cover Glasses (18x18mm)	Marienfeld, Nordrhein-Westfalen
Polystyrene Round-Bottom tubes (14ml)	Beckton Dickinson, NJ, USA
Reaction tubes (1.5 ml)	Sarstedt AG & Co Nümbrecht
Reaction tubes (2 ml)	Sarstedt AG & Co Nümbrecht
FACS tubes, flow cytometry	Sarstedt AG & Co, Nümbrecht
Pipette-tips, TipOne®, Graduated, Filter Tips	STARLAB GmbH, Hamburg
Pipette-tips, TipOne®, Graduated, Blue/Yellow/White	STARLAB GmbH, Hamburg
Pipette-tips with wide aperture	STARLAB GmbH, Hamburg
Counting Chamber Slides, Fast read 102™	Immune-systems, Paignton, UK
Glass beakers	SCHOTT AG, Mainz
Insulin syringes	B.Braun AG, Melsungen
FACS tubes	Sarstedt AG & Co, Nümbrecht
BD BioCoat™ Matrigel™ Invasion Chamber	BD Biosciences, Heidelberg

2.1.3 Chemicals and Media

The standard chemicals were obtained from AppliChem GmbH (Darmstadt, Germany), ROTH ® (Karlsruhe) and SIGMA-ALDRICH ® (Munich).

Description	Company
4′.6-Diamidin-2-phenylindol (DAPI)	Molecular Probes, Eugen, OR, USA
3D IrECM (Cultrex™)	AMS Bio, Abingdon, UK
Agarose	ROTH®, Karlsruhe
Albumin Fraction V (pH 7)	AppliChem GmbH, Darmstadt
Ammonium peroxodisulfate (APS)	ROTH®, Karlsruhe
Adefo Citroline 2000 (Developer)	ADEFO-CHEMIE GmbH, Dietzenbach
Adefofix (Fixer)	ADEFO-CHEMIE GmbH, Dietzenbach
Bromophenol blue	AppliChem GmbH, Darmstadt
Bovine Serum Albumin (BSA)	AppliChem GmbH, Darmstadt
Dichloroacetic acid (DCA)	AppliChem GmbH, Darmstadt
Deoxynucleotides (dNTP) Mix (high conc.)	New Englands Biolabs Inc, Frankfurt
Dulbecco´s Phosphate Buffered Saline (PBS)	PAA Laboratories GmbH, Pasching, Austria
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen™, Darmstadt
Dithiothreitol (DTT)	SIGMA-ALDRICH®, Hamburg
Ethanol	SIGMA-ALDRICH®, Hamburg
Ethylenediaminetetraacetic acid (EDTA)	AppliChem GmbH, Darmstadt
Ethidium bromide (EtBr)	ROTH®, Karlsruhe
FACSFlow	Becton DICKINSON, Heidelberg

Foetal Calf Serum Gold (A15-151)	PAA Laboratories GmbH, Pasching, Austria
Geneticin (G418)	Applichem GmbH, Darmstadt
Glycine	Applichem GmbH, Darmstadt
Glycerine	ROTH®, Karlsruhe
Halt™ Protease Inhibitor Single-Use Cocktail	Thermo Fisher Scientific, Dreieich
Hydrogen chloride (HCl)	AppliChem GmbH, Darmstadt
Isopropanol	SIGMA-ALDRICH®, Hamburg
Kanamycin	ROTH®, Karlsruhe
Lysogeny broth (LB) medium	ROTH®, Karlsruhe
LB agar	ROTH®, Karlsruhe
Methylene blue C.I. 52015	AppliChem GmbH, Darmstadt
Methanol	SIGMA-ALDRICH®, Hamburg
Milk Powder	ROTH®, Karlsruhe
RNAse/DNase-free water	Invitrogen™, Darmstadt
Rubidium Chloride	SIGMA-ALDRICH®, Hamburg
Sodium Hydroxide (NaOH)	SIGMA-ALDRICH®, Hamburg
Sodium Chloride (NaCl)	SIGMA-ALDRICH®, Hamburg
Sodium dodecylsulfate (SDS)	ROTH®, Karlsruhe
Non Reducing Lane Marker, Sample Buffer	Thermo Fisher Scientific, Dreieich
OPTI-MEM®	Invitrogen™, Darmstadt
Penicillin / Streptomycin (5 U ml^{-1})	Invitrogen™, Darmstadt
Pierce® ECL, Western Blotting Substrate	Thermo Scientific, Karlsruhe
Ponceau S	AppliChem GmbH, Darmstadt

ProSieve® QuadColor™ Protein Marker	Lonza, Cologne
Propidium Iodide	Invitrogen™, Darmstadt
Protein G Agarose Beads P7700	SIGMA-ALDRICH®, Hamburg
Restore™ Western Blot Stripping Buffer	Thermo Scientific, Karlsruhe
Ribonuclease A (RNase A; 100 mg ml^{-1})	QIAGEN, Hilden
Roti-Fect PLUS	ROTH®, Karlsruhe
Rotiphoresis gel 30	ROTH®, Karlsruhe
SDS-Pellets	ROTH®, Karlsruhe
Triton X-100	AppliChem GmbH, Darmstadt
Sepharose A/G Protein Beads	GE Healthcare, Buckinghamshire, UK
Silicon for cloning cylinders	Momentive performance materials, Albany, NY, USA
Tetramethylethylenediamin (TEMED)	ROTH®, Karlsruhe
Trypan Blue Stain 0.4 %	Invitrogen™, Darmstadt
Trypsin/Ethylene diamine tetraacetic acid (EDTA, 0.25 %)	Invitrogen™, Darmstadt
Tween® 20	AppliChem GmbH, Darmstadt
Tris hydroxymethyl aminomethane (Tris)	ROTH®, Karlsruhe
Triton X-100	AppliChem GmbH, Darmstadt
Vectashield® Mounting Medium	Vector, Burlingame, CA, USA

2.1.4 Solutions and Buffers

4'.6-Diamidine-2-phenylindole (DAPI)-Staining

DAPI concentration	300 ng/ml
Fixing Solution	3.7% Formaldehyde in PBS

Triton X-Solution	0.25% Triton X-100 in PBS	

Cell Lysis

Radio-Immunoprecipitation Assay (RIPA)-Buffer

RIPA-Buffer (10x)	150 mM	NaCl
	50 mM	Tris, pH 8.0
	1 %	Triton X-100
	0.5 %	Dichloroacetic acid (DCA)
	0.1 %	SDS

Polyacrylamide Gel Electrophoresis and Western Blot

1M Tris HCl (pH 8.8)	60.6 g Tris	
	Adjust volume to 500 ml with distilled water	
	pH 8.8 with HCl	
Electrophor. Buffer	25.0 mM	Tris
	191 mM	Glycine
	3.47 mM	SDS
	Adjust volume to 1 L with distilled water	
TBS (10x)	87.7 g	NaCl
(pH 7.5)	12.1 g	Tris
	Adjust volume to 1 L with distilled water	
TBS-T	100 ml	TBS (10x)
(pH 7.5)	0.1 %	Tween 20
	Adjust volume to 1 L with distilled water	
Milk Powder Solution	5 % (w/v)	Milk powder in TBS-T
Ponceau	65.7 µM	Ponceau S
	37.5 ml	Trichloroacetic acid (TCA)
	Adjust volume to 250 ml with distilled water	
Reducing Electrophoresis Buffer	5 ml 50% Glycerol	
	0.9255 g 0.6 M DTT (Mr = 154.25)	
	1.028 g 10.28% SDS	
	3.5 ml 0.35 M Tris pH6.8	
	1.2 mg 0.012% Bromophenol bleu	

Adjust volume to 10 ml with distilled water, 2 ml aliquots (-20°C)

Transfer Buffer
3.03 g Tris
12.1 g Glycine
20 % Methanol
0.05 % SDS
Adjust volume to 1 L with distilled water

TAE (Tris-acetate) Buffer 50x
242 g Tris in 500 ml distilled water
100 ml 0.5 M Na_2EDTA (pH 8.0)
57.1 ml glacial acetic acid
Adjust volume to 1 L with distilled water

LB medium
20 g LB medium
1 L distilled water

LB agar plates
35g LB agar
1 L distilled water

DNA loading dye
5x Blue Run (AGS)
for 10 ml
0.25 ml 1 M Tris/HCl pH 7
3 ml 0.5 M EDTA
5 mg Bromophenol bleu
2.5 ml Glycerol
4.25 ml distilled water

Table 1: Pipetting scheme for two discontinuous SDS-Electrophoresis gels (8.3 cm x 7.3 cm x 1 mm)

	Separation gel 10 %	Separation gel 12 %	Collection gel 5 %
Distilled Water	4.7 ml	3.6 ml	5.5 ml
Rotiphoresis Gel 30	5.4 ml	6.5 ml	836 µl
Tris HCl (pH 8.8)	6.0 ml	6.0 ml	-
Tris HCl (pH 6.8)	-	-	626 µl
10 % SDS	162 µl	162 µl	50 µl
20 % APS	54 µl	54 µl	40 µl
TEMED	12 µl	12 µl	5 µl

2.1.5 Antibodies

Table 2: Characteristics of primary antibodies used for western blotting, immunoprecipitation and immunofluorescence staining assays

Antibody	Host	Under -class	Stock Solution [µg ml^{-1}]	Dilution	Molecular weight [kDa]	Company	Catalogue number
Anti-β-Actin	rabbit	IgG	1000	1:10 000	42	SIGMA-ALDRICH, Hamburg	A5441
Anti-Lamin B1	mouse	IgG1-Kappa	200	1:200	68	MBL, MA, USA	JM-3046-100
Anti-Calnexin	mouse	IgG	250	1:500	90	BD Biosciences, NJ, USA	610524
Anti-Survivin	rabbit	IgG	200	1:750	16.5	R&D Systems, Heidelberg	AF886
Anti-phospho-H2A.X (Ser139)	mouse	IgG1	1000	1:1000	17	Millipore, MA, USA	05-636
Anti-53BP1	rabbit	IgG	1	1:1000	250	Novus Biologicals, UK	NB100-304
Anti-GFP	mouse	IgG	2000	1:1000	27	Abcam, Cambridge, UK	Ab1218
Anti-GFP	rabbit	IgG	2000	1:1000	27	Abcam, Cambridge, UK	Ab290
Anti-DNA-PKcs	mouse	IgG	500	1:250	450	BD Biosciences, NJ, USA	556456
Anti-DNA-PKcs	rabbit	IgG	200	1:1000/ 1:100	469	Abcam, Cambridge, UK	Ab70250
Isotype Control	rabbit	IgG	2500	1:100	-	Cell Signaling, MA, USA	3900
Isotype Control	mouse	IgG	2500	1:100	-	Cell Signaling, MA, USA	5415

Table 3: Characteristics of secondary antibodies used for immunofluorescence staining

Antibody	Host	Under -class	Stock Solution [mg ml^{-1}]	Dilution	Label	Company	Catalogue number
Anti-sheep IgG	donkey	IgG	2	1:250	Alexa Fluor^{R} 488	Molecular Probes, USA	A-11015
Anti-sheep IgG	donkey	IgG	2	1:250	Alexa Fluor^{R} 594	Molecular Probes, USA	A-11016

Table 4: Characteristics of secondary antibodies used for western blotting

Coupled enzyme	Specificity	Host	Under-class	Stock solution [µg ml^{-1}]	Dilution	Company	Catalogue number
Horse radish peroxidase	rabbit	goat	IgG	400	1:4000	Santa Cruz Biotechnology, Heidelberg	Sc-2254
Horse radish peroxidase	mouse	goat	IgG	400	1:1000		Sc-2255

2.1.6 Expression plasmids

Survivin expression plasmids:
pEGFP-N1 control plasmid, Clontech, Mountain View, CA, USA
(EGFP = Enhanced Green Fluorescent Protein)

pEGFP-N1-Survivin wt (wildtype); made by Dr. S. Hehlgans, C. Petraki, Molecular Radiobiology, University Hospital Frankfurt

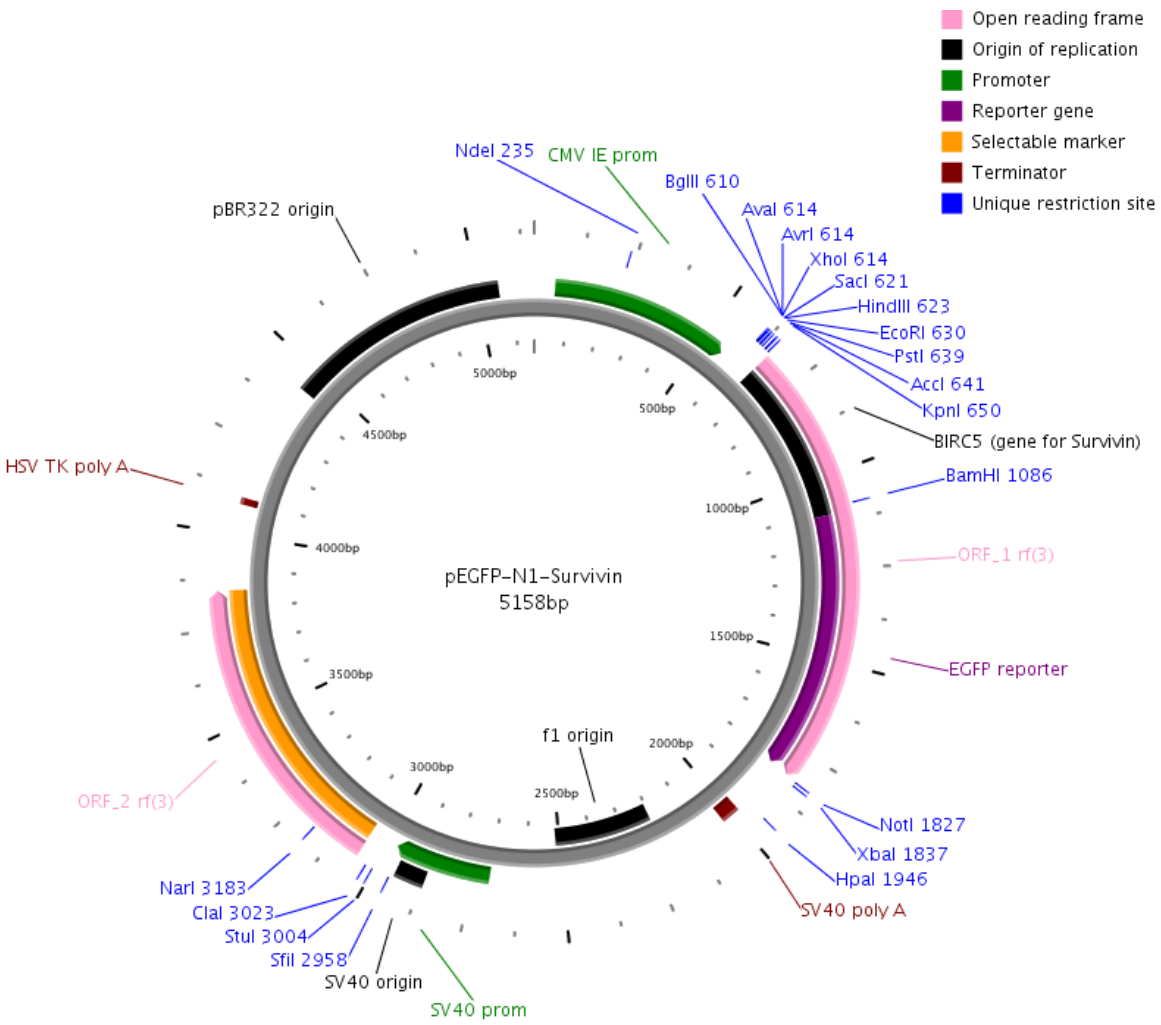


Figure 4: Plasmid map for pEGFP-N1-wild type Survivin. Plasmid map for pEGFP-N1-Survivin: The map of the 5158 bp long plasmid shows „*open reading frames*“ (in pink), „*origins of replication*“ (in black), promoters (in green), terminators (in brown), restriction sites (in blue), a resistance gene against Kan/Neo (in orange) and the EGFP reporter gene (in purple) as well as the BIRC5 gene which codes for Survivin. The plasmid map was created by *PlasMapper*.

The pEGFP-N1 vector was used to generate the Survivin-pEGFP fusion constructs, and the plasmid map shows an example of the wild type Survivin-pEGFP (Figure 4).

2.1.7 Specific small interfering RNA

<u>Survivin specific small interfering RNA (siRNA)</u>

Negative control siRNA	QIAGEN, Hilden
BIRC5 Survivin 3 siRNA (#s1458)	Ambion, Austin, TX, USA

Sequence (5' → 3')	Sense:	GCAGGUUCCUUAUCUGUCAtt
	Antisense:	UGACAGAUAAGGAACCUGCag

2.1.8 Commercial Kits

Description	Company
Micro BCA™ Protein Assay Kit	Thermo Scientific, Karlsruhe
PCR Mycoplasma Test Kit	AppliChem GmbH, Darmstadt
QIAquick Gel Extraction Kit	QIAGEN GmbH, Hilden
QIAquick PCR Purification Kit	QIAGEN GmbH, Hilden
NucleoBond Xtra Midi Plus EF	Macherey-Nagel GmbH &co. KG, Dueren
QIAprep Spin Miniprep Kit (250)	Qiagen GmbH, Hilden
PCR clean-up NucleoSpin® Extract II	Macherey-Nagel GmbH & co. KG, Dueren
Nuclear Complex Co-IP Kit	Active Motif, Belgium
QuikChange II Site-Directed Mutagenesis Kit	Agilent Technologies, La Jolla CA, USA
Caspase-Glo® 3/7 Assay	Promega, WI, USA

2.1.9 Enzymes and respective buffers

Description	Company
PfuUltra® High-Fidelity DNA Polymerase	Strategene, La Jolla, USA
T4-DNA-Ligase	New Englands Biolabs Inc, Frankfurt
T4 DNA Ligase buffer 10x	New Englands Biolabs Inc, Frankfurt
Taq-DNA-Polymerase	SIGMA Chemical Co, St. Louis, USA
DpnI	New Englands Biolabs Inc, Frankfurt
KpnI	New Englands Biolabs Inc, Frankfurt

XbaI	New Englands Biolabs Inc, Frankfurt
BamHI	New Englands Biolabs Inc, Frankfurt
EcoRI	New Englands Biolabs Inc, Frankfurt
ApaLI	New Englands Biolabs Inc, Frankfurt
FspI	New Englands Biolabs Inc, Frankfurt
NEB Buffer 10x	New Englands Biolabs Inc, Frankfurt

2.1.10 Electrophoresis Markers

Description	Company
100bp DNA-Ladder	Invitrogen™, Gaithersburg, USA
1kb DNA-Ladder	Invitrogen™, Gaithersburg, USA

2.1.11 Oligonucleotides

The melting temperature of all the oligonucleotides used was calculated according to this formula: T_m = 4°C x (G + C) + 2°C x (A + T) from Nelson and Brutlag, 1979. They were provided by Eurofins MWG Operon (Ebersberg, Germany).

Primers for PCR cloning

Primers for subcloning of Survivin from pc3-Survivin-GFP into pEGFP-N1

- **pEGFPC1-fw:** GAT CAC TCT CGG CAT GGA C
- **pEGFPC1-rev:** CAT TTT ATG TTT CAG GTT CAG GG
- **CMV-fw:** CGC AAA TGG GCG GTA GGC GTG
- **pEGFPN1-rev:** GTC CAG CTC GAC CAG GAT G

Primers for amplification of Survivin and subcloning in pEGFP-N1/KpnI/BamHI

- **1-Surv-fw:** gg-ggtacc-ggcggc-ATGGGTGCCCCGACGTTGC, KpnI, T_m = 68°C
- **2-Surv-rev:** cg-ggatcc-cg-ATCCATGGCAGCCAGCTGCTC, BamHI, T_m = 68 °C

Primers for Survivin S20A mutation

- **5-S20A-fw:** 5'-tcaaggaccaccgcatcgctacattcaagaactgg-3', T_m = 79.27°C
- **6-S20A-rev:** 5'-ccagttcttgaatgtagcgatgcggtggtccttga-3', T_m = 79.27°C

Primers for Survivin S20D mutation

- **7-S20D-fw:** 5'-ctcaaggaccaccgcatcgatacattcaagaactggcc-3', T_m = 78.97°C
- **8-S20D-rev:** 5'-ggccagttcttgaatgtatcgatgcggtggtccttgag-3', T_m = 78.97°C

Primers for Survivin T34A mutation

- **9-T34A-fw:** 5'-gctgcgcctgcgccccggagcgg-3, T_m = 81.67°C
- **10-T34A-rev:** 5'-ccgctccggggcgcaggcgcagc-3', T_m = 81.67°C

Primers for Survivin T34D mutation

- **11-T34-fw:** 5'-gggctgcgcctgcgacccggagcggatg-3', T_m = 81°C
- **12-T34D-rev:** 5'-catccgctccgggtcgcaggcgcagccc-3', T_m = 81°C

Primers for Survivin T117A mutation

- **13-T117A-fw:** 5'-gccaagaacaaaattgcaaaggaagccaacaataagaagaaagaat-3', T_m = 78.02°C
- **14-T117A-rev:** 5'-attctttcttcttattgttggcttcctttgcaattttgttcttggc-3', T_m = 78.02°C

Primers for Survivin T117D mutation

- **15-T117D-fw:** 5'-gagccaagaacaaaattgcaaaggaagacaacaataagaagaaagaatttgagg-3', T_m = 78.96°C
- **16-T117D-rev:** 5'-cctcaaattctttcttcttattgttgtcttcctttgcaattttgttcttggctc-3', T_m = 78.96°C

Primers for Survivin ΔXIAP mutation

For the 5' end of deletion mutants XIAP and BIR primers 19 and 20 were annealed and subsequently used for ligation reactions.

- **19- XIAP-fw:** starts with a.a.1 to 14, KpnI C-ATG GGT GCC CCG ACG TTG CCC CCT GCC TGG CAG CCC TTT CTC - T XbaI
- **20- XIAP-rev:** starts with a.a. 14 to 1, XbaI CTAGA- GAG AAA GGG CTG CCA GGC AGG GGG CAA CGT CGG GGC ACC CAT – GGTAC KpnI

- **21-XIAP-39fw**: starts with a.a.39, XbaI GC-TCT AGA-GCC GAG GCT GGC TTC ATC CA, T_m = 66°C
- For reverse primer see primer no. **2-Surv-rev**

Primers for Survivin ΔBIR mutation

- For BIR domain deletion for the 5'end refer to primers no. **19-XIAP-fw** and **20-XIAP-rev**
- **22-BIR-fw**: starts with a.a. 88, XbaI GC-TCT AGA-TCT GTC AAG AAG CAG TTT GAA GA, T_m =64°C
- For reverse primer see primer no. **2-Surv-rev**

Primers for Survivin ΔMicTub mutation

- For fw primer see primer no. **1-Surv-fw**
- **23-Mictub-rev:** starts with a.a. 98 BamHI cg-ggatcc-AAG GGT TAA TTC TTC AAA CTG CTT, T_m = 64°C

Primers for Survivin ΔHsp90 mutation

- For fw primer see primer no. **1-Surv-fw**
- **24-Hsp90-rev:** starts with a.a. 78 XbaI GC-TCT AGA-TTT ATG TTC CTC TAT GGG GTC G,T_m = 64°C
- For the amplification of the 3'end of Hsp90 deletion mutant primer no. **22-BIR-fw** and **2-Surv-rev** were used

Primers for sequencing

- **37-CMV-fw-1:** CGTAACAACTCCGCCCCATTG, T_m = 66°C
- **38-pEGFPN1-rev-1:** CTTGTGGCCGTTTACGTCGC, T_m = 64°C

2.2 Methods

2.2.1 Cell culture

The human colorectal carcinoma cell line SW480 was obtained from the American Type Culture Collection (ATCC, Promochem, Germany). Cells were cultured in Dulbecco's Modified Eagle Medium containing glutamax-I (L-alanyl-L-glutamine) and supplemented with 10% foetal calf serum, 50 U/ml penicillin and 50 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Cells were passaged on average 1:6 every 3 to 4 days using 2 ml trypsin after being washed with PBS (see *2.1.3 chemicals and media* section) and plated on T-75 flasks with 12 ml of the medium mentioned above. In the case of the Survivin-EGFP constructs 750 µg/ml of G418 was added to the medium.

For 3D cell cultures, cells were plated into a mixture of 0.5 mg/ml laminin-rich extracellular matrix (lrECM; Cultrex 3D Culture Matrix BME Reduced Growth Factor Basement Membrane Extract; R&D Systems, Wiesbaden, Germany) and DMEM medium as previously described, on either 96-well plates (for 3D colony forming assays) or 24-well plates (for 3D lysates and 3D immunofluorescence staining assays).

2.2.2 Survivin constructs and stable transfection

For expression of Survivin-EGFP fusion constructs human Survivin cDNA was amplified with specific primers (listed in section *2.1.11*), flanked with KpnI and BamHI restriction sites, from an expression plasmid provided by R.H. Stauber (University Hospital of Mainz, Germany). Subsequently, the PCR fragments were digested with appropriate restriction enzymes (see section *2.1.9*) and were inserted into KpnI/BamHI sites of the pEGFP-N1 expression vector. For deletion mutants, cDNA fragments left and right from the deletion were amplified by PCR

and ligated using the XbaI restriction site while inserted into the KpnI/BamHI restriction sites of the pEGFP-N1 vector. The phospho-mutants were generated using the site-directed mutagenesis kit (section *2.1.8*) according to manufacturer's guidelines using the primers listed in section *2.1.11*.

PCR for amplification of Survivin cDNA for subcloning in pEGFP-N1

pc3-Surv-GFP was used as a template at a working concentration of 50 ng/µl along with (per reaction): 30µl 10 x Buffer, 7.5 µl of 10µM primers 1 and 2, respectively (for primer names see section *2.1.11*), 7.5 µl of 10 mM dNTPs, 229.5 µl RNase/DNase-free water and 6 µl PfuUltra HF. The PCR conditions were as follows: 2 min 95°C, 35 x (30 s 95°C, 1 min 62°C, 1 min 72°C), 15 min 72°C, 4°C. The samples were purified using the QIAquick PCR Purification Kit following manufacturer's instructions.

Restriction enzyme digestion of Survivin PCR fragment and pEGFP-N1

For restriction enzyme digestion 10 µg of the pEGFP-N1 plasmid or 10 µg of the Survivin PCR fragment were used for digestion with: 3 µl KpnI + 3 µl BamHI + 1 µl BSA + 10 µl NEBuffer #1. The reaction volumes were adjusted to 100 µl with RNase/DNase-free water. The samples were incubated overnight at 37°C.

Gel Purification of plasmid pEGFP-N1

50 ml of 1% agarose gel (supplemented with 3 µl EtBr) in TAE buffer was used (in a gel electrophoresis chamber) for the loading of 120 µl KpnI/BamHI linearized plasmid for gel electrophoresis at 80 Volts for 1 hour. The plasmid was then extracted using the QIAquick Gel Extraction kit following manufacturer's instructions.

Ligation of Survivin with the pEGFP-N1

Ligation reaction contained the plasmid DNA pEGFP-N1/KpnI/BamHI (2 µl), the insert Survivin/KpnI/BamHI (2 µl), T4 DNA Ligase (1µl), 10 x T4 DNA Ligase Buffer (NEB) (1 µl) and RNase/DNase-free water (4 µl), followed by overnight incubation at 14°C.

PCR for Survivin-EGFP deletion mutants

Survivin cDNA was used as a template at a 50 ng/µl working concentration. For the PCR reaction forward and reverse primers (for primer names see section *2.1.11*) (number 21/2 for ΔXIAP right, 22/2 for ΔBIR right, 1/28 for ΔMicTub left, 1/24 for ΔHsp90 left and 22/2 for ΔHsp90 right) at a working concentration of 10 µM (1.25 µl each primer per reaction), 10x NEBuffer (5 µl per reaction), dNTPs 10 mM (1.25 µl per reaction), RNase/DNase-free water (38.25 µl per reaction) and PfuUltra HF (1 µl per reaction) were added. The PCR conditions were as follows: 2 min 95°C, 35 x (30 s 95°C, 1 min 62°C, 1 min 72°C), 15 min 72°C, 4°C. Agarose gel analysis followed (via agarose gel electrophoresis).

Restriction enzyme digestion of Survivin deletion mutants PCR fragments

45 µl of each PCR reaction was digested with 3 µl of each restriction enzyme + 10 µl NEBuffer #2 or #3 (ΔXIAP right: XbaI/BamHI/#3, ΔBIR right: XbaI/BamHI/#3, ΔMicTub left: KpnI/BamHI/#3, ΔHsp90 left: KpnI/XbaI/#2, ΔHsp90 right: XbaI/BamHI/#3) + 1 µl BSA + 38 µl RNase/DNase-free water. The samples were incubated overnight at 37°C.

PCR clean-up

Samples were purified using the PCR clean-up NucleoSpin^{R} Extract II kit following manufacturer's protocol.

Ligation reactions for Survivin deletion mutants

ΔXIAP: 2µl pEGFP-N1/KpnI/BamHI, 1µl annealed primers 19 and 20/KpnI/XbaI, 2 µl Surv. ΔXIAP right/XbaI/BamHI, 1 µl T4 DNA Ligase, 1 µl 10 x T4 DNA Ligase Buffer (NEB), 3 µl RNase/DNase-free water

ΔBIR: 2µl pEGFP-N1/KpnI/BamHI, 1 µl annealed primers 19 and 20/KpnI/XbaI; 2.5 µl Surv ΔBIR right/XbaI/BamHI, 1 µl T4 DNA Ligase, 1 µl 10 x T4 DNA Ligase Buffer (NEB), 2.5 µl RNase/DNase-free water

ΔMicTub: 2µl pEGFP-N1/KpnI/BamHI, 2 µl ΔSurv MicTub left/KpnI/BamHI, 1 µl T4 DNA Ligase, 1 µl 10 x T4 DNA Ligase Buffer (NEB), 4 µl RNase/DNase-free water

ΔHsp90: 2µl pEGFP-N1/KpnI/BamHI, 2.5 µl Surv ΔBIR right/XbaI/BamHI, 3 µl Surv ΔHsp90 left/KpnI/XbaI, 1 µl 10 x T4 DNA Ligase Buffer (NEB), 0.5 µl RNase/DNase-free water

0.2 ml PCR tubes were used for the reactions which were incubated overnight at 14°C.

Transformation

50 µl of competent TOP10 E.coli stored with Rubidium Chloride were used for each transformation required (everything kept on ice). 3 µl of Dpn I digested DNA was added to 50 µl bacteria (1.5 ml Eppendorf tubes were used) and the tubes were incubated for 20 minutes on ice before they were subjected to a heat shock at 42°C for 45 seconds and then placed back on ice for 2 minutes. Then, 250 µl of LB-Medium (warmed at 37°C) was added to each tube. All samples were incubated at 37°C for 1 hour while being shaked at 250 rpm. Subsequently, samples were centrifuged at 3,000 rpm for 3 minutes (RT). 200 µl of supernatants were removed and the pellets were resuspended in the medium that was left.

The samples were then spread on LB-covered dishes (with 50 µg/ml Kanamycin) and incubated overnight at 37°C followed by storage at 4°C.

4 ml overnight culture of bacteria

Using 50 ml tubes, 50 ml of LB medium was mixed with 50 µl of Kanamycin and each 4 ml of this mixture were transferred to 14 ml polystyrene round-bottom tubes (one tube per clone). Using a 200 µl pipette-tip, *E. coli* colonies were picked from the LB-covered dishes (following transformation) and were placed into the round-bottom tubes that were then overnight incubated at 37°C while being shaken at 250 rpm followed by plasmid mini-preparation.

Plasmid mini-preparation

After transformation of TOP10 bacteria and subsequent inoculation of selected colonies plasmid mini preparation was performed using the *QIAprep Spin Miniprep* kit following the manufacturer's instructions. The solutions provided by the kit were the following: Buffer P1 (for resuspension) contained 50 mM Tris/HCl, pH 8.0, 10 mM EDTA and 100 µg/ml Rnase A; buffer P2 (for lysis) contained 200 mM NaOH, 1% SDS; buffer N3 (for neutralisation) contained 3M KOAc, pH 5.5. Two ml from the content of each round-bottom tube (having been inoculated) were transferred to 2 ml Eppendorf tubes that were centrifuged at 5,000 rpm (tabletop centrifuge) for 5 minutes (RT). Supernatants were discarded and 250 µl of buffer P1 was added in each pellet followed by vortexing. Then, 250 µl of buffer P2 was added to the tubes, the contents were mixed and 350 µl of buffer N3 was also added to each tube, mixed and centrifuged at 13,000 rpm (tabletop centrifuge) for 10 minutes (RT). All supernatants (now containing plasmid-DNA) were transferred to Qiaprep spin columns and centrifuged at 13,000 rpm for 1 minute; all eluates were discarded. The columns were washed with 0.5 ml buffer PB (provided by the kit) and centrifuged at 13,000 rpm for 1 minute; eluates were discarded. The columns were washed again this time with 0.75 ml buffer PE and centrifuged at same settings; eluates were discarded. The empty columns

were dry-centrifuged at the same settings. Subsequently, the columns were placed in new 1.5 ml Eppendorf tubes when 50 µl of buffer EB (for elution – containing 10 mM TrisCl, pH 8.5) was added to each of the column, incubated for 1 minute and centrifuged at same settings. The concentration was measuered in an Eppendorf Biophotometer (diluting each plasmid DNA 1:100 with water).

Plasmid midi-preparation

When certain clones per construct were confirmed by sequencing (Eurofins, MWG Operon), they were followed by midi preparation using the *NucleoBond Xtra Midi Plus EF* kit according to manufacturer's guidelines. Prior to preparation, the according inoculation was first carried out. In detail, in a 500 ml glass beaker, 100 ml of LB medium was supplemented with 50 µg/ml Kanamycin and inoculated with 100 µl overnight cultures (dilution 1:1000) at 37°C and 175 rpm shaking. On the following day, 50 ml of each of these cultures were transferred to 50 ml tubes. The bacteria were pelleted by centrifugation at 4,000 rpm for 15 minutes at 4°C. Supernatants were discarded and 8 ml of RES-EF (provided by the kit) was used for resuspension. Eight ml of Lys-EF was added, mixed and incubated at RT for 5 minutes. 8 ml of NEU-EF was then added, mixed and incubated on ice for 5 minutes. Subsequently, 15 ml of EQU-EF was carefully added to the column filters and the lysates were then mixed and poured into the columns which were then washed with 5 ml FIL-EF before the paper filters were removed. The columns were washed with 35 ml ENDO-EF followed by washing with 15 ml WASH-EF before they were put in new 50 ml tubes in order to elute the plasmid-DNA with 5 ml ELU-EF. The columns were removed; 3.5 ml of Isopropanol was added to the eluates, mixed and incubated at RT for 2 minutes before they were syringed through a NucleuoBond Finalizer in a 30 ml-syringe twice and washed 1 x with 2 ml 70% EtOH (Endotoxin-free from kit). Then, the NucleoBond Finalizer was placed in a 1 ml-syringe and 0.5 ml H_2O-EF was used to collect the eluate in 1.5 ml Eppendorf tubes. Concentrations were determined using a Bio photometer.

Stable transfection

Prior to stable transfection, the constructs were linearized using 30 µg of plasmid DNA, 10 µl of 10 x NEBuffer, 4 µl of ApaLI enzyme (10 U/µl), 1 µl BSA and the volumes were adjusted to 100 µl with RNase/DNase-free water while the reactions were set up in 1.5 ml tubes incubated overnight at 37°C. After the confirmation of the linearization via agarose gel electrophoresis, the linearized plasmids were subjected to PCR clean-up using the NucleoSpin Extract II kit following manufacturer's instructions. SW480 cells were then stably transfected with pEGFP-N1 or Survivin-EGFP expression constructs using 4 µg linearized plasmid DNA diluted in serum free Opti-MEM and *Roti-Fect PLUS* transfection reagent according to manufacturer's instructions (see section *2.2.3* for method of transfection). Following transfection, the cells were transferred from 6-well plates to 100 mm petri dishes where colonies were allowed to grow in DMEM + 10% FCS +1% P/S enriched with 750 µg/ml G418 for selection. Clones were isolated using cloning cylinders embedded in silicon while expression of EGFP or Survivin-EGFP constructs was verified by fluorescence microscopy and Western blotting.

Selection of fluorescent colonies

When the 100 mm petri dishes (mentioned above) contained colonies large enough to be visible by naked eye, the dishes were washed with PBS and transferred to a fluorescence microscope where fluorescent colonies were selected and marked (from the outside of the dish). The dishes were then taken under laminar flow hood where silicon-embedded cloning cylinders were carefully placed on the selected colonies and 60 µl of trypsin was added to each cloning cylinder for trypsinization (5 minutes incubation at 37°C/5%CO_2 under sterile conditions). The cells were then resuspended in the cylinders, with 60 µl of DMEM + 10% FCS +1% P/S and transferred in 12-well plates (one clone per well) along with 1 ml medium (DMEM + 10% FCS +1% P/S + 750 µg/ml G418) per well. They were then left in those 12-

well plates to grow to approximately 60-80% confluence before their transfer to T-25 flasks. Cells from T-25 flasks were later (when 80-100% confluent) harvested for cryostocks.

Fluorescence imaging

Fluorescence images of stably transfected cells were obtained using an Axiovert 40 CFL microscope (Carl Zeiss, Göttingen, Germany) after staining the nuclei with 4',6-diamidino-2-phenylindole (DAPI).

2.2.3 Attenuation of endogenous Survivin via siRNA

For the knockdown of endogenous Survivin, an siRNA targeting the 3'-untranslated region of Survivin was used: Surv. siRNA: 5'- GCAGGUUCCUUAUCUGUCAtt-3' (sense), siRNA ID s1458. The non-specific negative control siRNA was from Qiagen. 24 hours after plating 3.5 x 10^5 cells of each of the Survivin-EGFP constructs on 6-well plates, both of the siRNAs were diluted in serum free Opti-MEM to obtain a final concentration of 20 nM, in this case 2.5 µl siRNA (10µM) plus 122.5 µl Opti-MEM calculated for each well. Roti-Fect PLUS transfection reagent was used under serum-free conditions at a concentration of 20 nM. Thus, Roti-Fect was also diluted in serum free Opti-MEM (5 µl Roti-Fect plus 120 µl Opti-MEM calculated per well) and 5 minutes later it was mixed with the diluted siRNA and incubated for 20 minutes at RT. In the meanwhile, cells were washed once with PBS and 1 ml of serum free Opti-MEM was added. Subsequently, the siRNA/Opti-MEM solution was mixed with the Roti-Fect/Opti-MEM solution, and 250 µl of the mixture was added to each well. The 6-well plates were incubated for 8 hours at 37°C, 5% CO_2, followed by addition of 1.25 ml Opti-MEM/20% FCS to each well. The 6-well plates were incubated for 24 hours at 37°C 5% CO_2 prior to any further experiments.

2.2.4 Irradiation procedure

Irradiation of cells with single photon doses ranging from 2 to 6 Gy was performed using a linear accelerator (SL-15, Elekta, Crawley, UK) with 6 MeV/100 cm focus-surface distance and a dose rate of 4 Gy/min. Mock-irradiated cultures were kept at room temperature in the X-ray control room while the other samples were being irradiated.

2.2.5 Three-dimensional colony forming assay

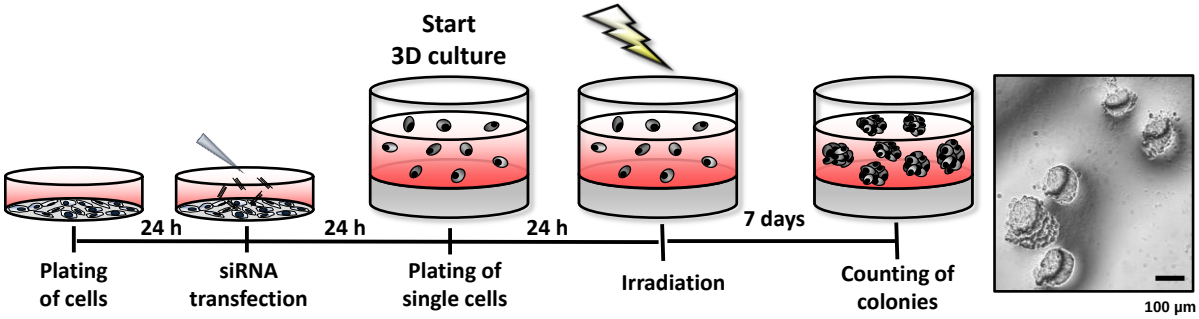


Figure 5: Methodology for 3D radiation clonogenic survival assays. The procedure of a 3D radiation clonogenic survival assay is briefly depicted here on a symbolic time scale followed by a photograph of colonies taken under the microscope (2.5x).

24 h after the knockdown of endogenous Survivin via siRNA transfection (Figure 5), the cells were washed with PBS (1 ml per well) and trypsinized (0.25 ml per well) in order to be counted with a chamber counter (1:2 dilution with trypan blue) under the microscope. Single cells were plated in 0.5 mg/ml lrECM/DMEM/10% FCS/1% penicillin/streptomycin on 96-well plates pre-coated with 50 µl 1% Agarose, by triplets (3 wells per construct per siRNA and 1 x 10^3 cells per well) in 100 µl cell-matrigel mixture. 200 µl of PBS was added to the wells around those containing cells. The 96-well plates were incubated for 90 minutes at 37°C, 5% CO_2, followed by addition of 100 µl of DMEM+10% FCS+1%P/S to each well. The plates were incubated at 37%, 5% CO_2 for 24 hours until irradiation (0, 2, 4, 6 Gy) and cell colonies

(> 50 cells) were microscopically counted 7 days after plating (Figure 5). Plating efficiencies were calculated as numbers of colonies counted (0Gy) / numbers of cells plated. Surviving fractions were calculated as numbers of colonies counted / (numbers of cells plated × plating efficiency). Each point on survival curves represents the mean surviving fraction from 3-4 or more independent experiments. Survival variables α and β were fitted according to the linear quadratic equation SF = exp $[-\alpha \times D - \beta \times D^2]$ with D = dose using EXCEL® software (Microsoft). The radiation-induced cytotoxicity enhancement factor at 50% cell survival was calculated by transforming the above mentioned equation using α and β values of the individual survival curves.

2.2.6 Protein extraction and Western blotting

Protein extraction from 3D cultures

24 hours after the plating of mock- and siRNA-transfected cells in 0.5 µg/µl lrECM on 24-well plates pre-coated with 300 µl of 1% Agarose (1 x 10^6 cells per well), the matrigel was carefully removed and 130 µl of ice-cold RIPA (see section *2.1.4 solution and buffers*) was added to each well and incubated on ice for 30 minutes. The RIPA/cells mixture was transferred from the 24-well plates to 2 ml Eppendorf tubes, incubated on ice for 10 minutes followed by passing through a 30-G insulin syringe to optimize protein extraction. The samples were then incubated on ice for 30 minutes, centrifuged for 15 minutes at 4°C 14,000 rpm followed by transfer of supernatants to new 1.5 ml Eppendorf tubes. For the estimation of the lysate volume, all samples were filled with RIPA up to the same volume of that with the largest volume. Loading buffer was then added to all samples (1/5 of the volume of the largest volume-sample). The samples were then stored at - 80°C before Western blotting.

Western blotting

Western blotting was performed in the following cases: to confirm the knockdown of endogenous Survivin, to confirm the correct sizes and expression of Survivin-EGFP mutants, their relation to other proteins as well as to identify immunoprecipitated complexes. For these reasons, 10% or 12% gels were prepared for the SDS-gel electrophoresis (see section *2.1.4* table 1) where 20 - 30 µg of protein extract was loaded per lane. Prior to electrophoresis all samples were heated at 95°C for 10 minutes, shortly (1 minute) centrifuged at 11,000 rpm at 4°C and during electrophoresis (25 mA 400V per gel) 1 L of electrophoresis buffer was used (see section *2.1.4* for buffers) in the chamber. Following gel-electrophoresis, the gels were placed on 7 x 9 cm filter papers (see section *2.1.2*) soaked in transferring-buffer (see section *2.1.4*) then covered with blotting membranes (6 x 8 cm) followed by two more filter papers and transferred on a semi-dry blotting apparatus for protein transfer at 50 mA per gel for 150 – 210 min. Following blotting, to confirm correct protein transfer and equal loading the membranes were soaked in Ponceau S solution for 1 minute, subsequently distained with distilled water and then blocked in 5% milk powder/TBS-T at room temperature for 30 minutes until primary antibodies were applied that were diluted in 5% BSA/TBS-T prior to application and incubated with the respective membranes at 4°C overnight. The following primary antibodies were used (for more details see Table 2 and 4): anti-Survivin (AF886), Anti-Calnexin (BD610524), anti-GFP (ab290 and ab1218), anti-Lamin B1 (JM-3046-100), anti-DNA-PKcs (BD556456 and ab70250), anti-β-actin (A5441). For detection of proteins, horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (diluted in 5% milk powder/TBS-T) were used after 3 x washing of the membranes (using TBS-T). The membranes were then incubated with appropriate dilutions of secondary antibodies in 5% milk powder/TBS-T for 1 hour followed by 4 washing steps with TBS-T and one with TBS. The membranes were then incubated for 1 min with working solutions of

enhanced chemiluminescent (ECL) substrate (Thermo Scientific), placed in plastic sheet protectors and exposed to Hyperfilm ECL (GE Healthcare) prior to film development.

2.2.7 Subcellular fractionation and immunoprecipitation

Cytoplasmic and nuclear extracts were prepared with the *Nuclear Complex Co-IP* kit according to manufacturer's protocol (all buffers hereby mentioned regarding fractionation were supplied by the kit) and subjected to co-immunoprecipitation experiments and western blotting as described.

Subcellular fractionation

Four million cells were plated per T-75 flask, and 4 x T-75 flasks were used per construct in order to be subjected to fractionation and eventually co-IP. On the second day after plating, serum starvation was performed, replacing the usual medium (DMEM/10% FCS/1% P/S) with serum-free medium (DMEM without FCS) in all T-75 flasks. On the day following the serum starvation, the flasks were washed with ice-cold PBS and scraped with 1 ml 5% Phosphatase Inhibitor/PBS. The scraped cells were transferred to pre-chilled 15 ml tubes that were centrifuged at 1,500 rpm at 4°C for 5 minutes. Supernatants were discarded and each pellet was resuspended in 500 µl ice-cold Hypotonic Buffer (containing Hypotonic buffer, Phosphatase Inhibitor, Protease Inhibitor and double distilled water) and incubated on ice for 15 minutes. 25 µl of detergent (provided by the kit) was then added in each sample, all of which were then centrifuged at 12,000 rpm at 4°C for 30 seconds. All cytoplasmic fractions were then transferred to 1.5 ml Eppendorf tubes properly labelled for the cytoplasmic fractions. The pellets were resuspended in 100 µl digestion buffer (containing 0.5% 100 mM PMSF, 98.5% digestion buffer, 1% Protease Inhibitor Cocktail) and 0.5 µl of enzymatic shearing cocktail was added to all samples before they were incubated at 4°C for 90 minutes. Following incubation, 2 µl of 0.5M EDTA was added to each sample which were

then gently vortexed for 2 seconds and incubated for 5 minutes on ice before they were centrifuged at 12,000 rpm for 10 minutes. The supernatants were collected (i.e. the nuclear fractions) in new 1.5 ml Eppendorf tubes. Concentrations were determined using the *Micro BCA assay* kit according to manufacturer's instructions.

Immunoprecipitation

For co-immunoprecipitation, 500 µg of nuclear extract (per sample) resuspended in 500 µl IP-Low buffer (included in the Nuclear Complex *Co-IP* kit) were incubated overnight at 4°C with anti-GFP (ab1218) or anti-DNA-PKcs (BD556456) antibodies. A mouse (G3A1) mAb IgG1 Isotype control antibody served as a control. The final concentration of all antibodies in the nuclear lysates was 2 µg/ml. Following overnight incubation, 50 µl of slurry protein G agarose beads (Sigma-Aldrich) was added to the samples and incubated at 4°C for 1 hour while gently being rotated. Then, the probes were centrifuged at 5,500 rpm for 2 minutes, supernatants were discarded and samples were washed 3 times with 500 µl IP-High buffer (included also in the Co-IP kit) and once with PBS at 5,500 rpm for 2 min at 4°C before 30 µl RIPA and 8 µl loading dye were added to the beads-pellet. The samples were heated for 10 minutes at 95°C and centrifuged at 11,000 rpm for 50 sec prior to SDS-electrophoresis and eventually western blotting.

2.2.8 Immunofluorescence staining and imaging

Residual DNA double-strand breaks (DSB) of 3D and 2D cell cultures (plated on 24-well plates) were evaluated using the foci assay.

Two-dimensional immunofluorescence staining

24 hours after the knockdown of endogenous Survivin (via siRNA) on 6-well plates, cells were harvested and plated on 24-well plates with DMEM/10% FCS/1% P/S for 2D

immunofluorescence staining (IF). In detail, cover slips were placed on 24-well plates and 75 x 10^3 cells were plated per well, for control siRNA and Survivin-targeted siRNA per construct. 24 hours after plating, the cells were irradiated with 2 Gy and stained 40 minutes or 24 hours later (different plates were prepared for different time points) according to the time point of interest. Cells were then permeabilized and fixed using 0.25% Triton X-100 and 3.7% formaldehyde in PBS for 10 minutes at RT. Subsequently, blocking took place via incubation for 1 hour with 3% BSA in PBS followed by staining with anti-phospho-histone γH2AX and anti-53BP1 antibodies 1:1000 diluted in 3% BSA in PBS for 1 hour (50 µl solution per cover slip was used). Cells were then washed once with PBS and stained with secondary antibodies i.e. Alexa Fluor 594 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit diluted 1:500 (Invitrogen) for another hour in the dark, followed by 4',6-diamidino-2-phenylindole (DAPI; Roth) staining for 5 minutes. Cover slips were then transferred on microscopic slides after being covered with one drop of Vectashield mounting medium. γH2AX/53BP1 foci were microscopically counted from at least 50 cells per experimental condition.

Three-dimensional immunofluorescence staining

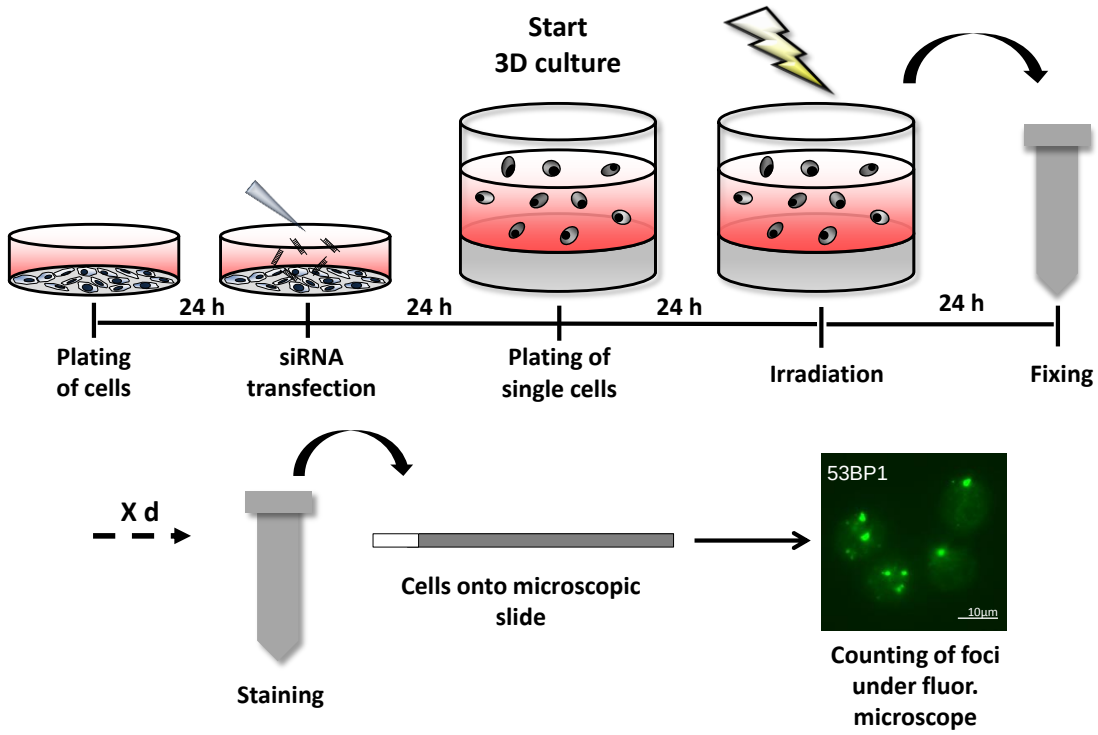


Figure 6: Methodology of 3D IF of γH2AX/53BP1 foci. The procedure of the 3D immunofluorescence staining of γH2AX/53BP1 foci for evaluation of residual DNA DSBs is hereby represented on a symbolic time scale followed by a photograph of nuclei taken under the microscope (40x). Bar: 10µm

24 hours after the siRNA-mediated attenuation of endogenous Survivin on 6-well plates (Figure 6), cells were harvested and plated on 24-well plates (pre-covered with 1% agarose) in a mixture of 0.5 mg/ml laminin-rich extracellular matrix and DMEM/10% FCS/1% P/S for 3D IF. In detail, 0.5 x 10^6 cells were plated per well, for control siRNA and Survivin-targeted siRNA per construct. 24 hours after plating, the cells were irradiated with 2 Gy. 24 hours following irradiation, the cells were transferred with the matrigel from the 24-well plates to 1.5 ml Eppendorf tubes and centrifuged for 3 minutes at 3,500 rpm (talbetop centrifuge). Supernatants were discarded and cells were permeabilized and fixed using 0.25% Triton X-100 and 3.7% formaldehyde in PBS (200 µl solution per tube) for 15 minutes at RT. Then,

the samples were centrifuged again at 4,500 rpm for 3 minutes, supernatants were removed, and the pellets were resuspended in 50 µl blocking solution (3% BSA in PBS) and left to be blocked for 1 hour. Subsequently, staining took place with 10 µl (per sample) of 1:500 diluted in blocking solution anti-phospho-histone γH2AX and anti-53BP1 (same dilution) antibodies for 2 hours. Cells were then washed once with PBS for 10 minutes, centrifuged at 4,500 rpm for 3 minutes and pellets were resuspended with 50 µl (per pellet) of 1:250 diluted in blocking solution secondary antibodies i.e. Alexa Fluor 594 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit and incubated for another hour in the dark. The cells were then washed twice with 1 ml PBS via resuspension followed by 10 minutes incubation and centrifugation at 4,500 rpm for 3 minutes. The pellets were finally resuspended in 15 µl DAPI solution (300 ng/µl) and incubated in the dark for 5 minutes. The cell suspensions were then transferred on microscopic slides, along with one drop of Vectashield mounting medium on each sample while a square cover slip was used to seal the cells. γH2AX and 53BP1 foci were microscopically counted from at least 50 cells per experimental condition (Figure 6).

Fluorescence imaging

Fluorescence images of γH2AX and 53BP1 foci were obtained using an Axiovert 40 CFL microscope (Carl Zeiss).

2.2.9 Cell cycle analysis

24 hours after the siRNA-mediated knockdown of endogenous Survivin, cells were harvested, counted and 3.5 x 10^5 cells were plated in triplets on 6-well plates with DMEM/10% FCS/1% P/S for cell cycle analysis. 24 hours after plating, the supernatants from all samples were transferred to 15 ml tubes. The cells were then washed with 5 ml PBS and trypsinized in 1 ml trypsin for 5 minutes at 37°C/5% CO_2. Cells were then resuspended in 3

ml medium and transferred in the 15 ml falcon tubes containing their supernatants. The tubes were then centrifuged at 1,000 rpm for 5 minutes, washed twice with 1 ml PBS, transferred to 1.5 ml Eppendorf tubes and centrifuged at 3,000 rpm for 5 minutes. Pellets were resuspended in 200 µl PBS and 1 ml 70% EtOH (cooled at -20°C) was added to each sample. They were then centrifuged at 2,000 rpm at 4°C for 5 minutes, each pellet was resuspended in 100 µl PBS followed by addition of 100 µl RNase A (100 µg/ml in PBS) and 100 µl propidium iodide (100 µg/ml in PBS). The samples were then incubated at 37°C for 30 min. Cell suspensions were then transferred in FACS tubes that were measured (12,000 events at 449 nm; FL2-585 Red) using the FACS Calibur flow cytometer and the *CellQuestPro* software (Becton Dickinson).

2.2.10 Measurement of apoptosis and caspase 3/7 assay

For quantification of apoptotic cells, subG1 content was analysed by flow cytometry as described in section *2.2.9*. For quantification of caspase 3/7 activity a CASPASE GLO™-assay was used according to manufacturer's instructions. In particular, 24 hours after the knockdown of endogenous Survivin (via siRNA on 6-well plates) cells were washed, trypsinized, counted and 6 x 10^3 cells were plated per well (in triplets per construct) on three 96-well plates (two for 0 Gy and one for 6 Gy) with DMEM/10% FCS/1% P/S. 24 hours later, one of the 0 Gy plates was measured after removing 70 µl medium from each well and adding 30 µl substrate (Caspase-Glo® 3/7 Assay Reagent), then shaking at 300 rpm for 30 seconds followed by 90 minutes incubation at RT. Measurement was conducted using an ELISA-Reader (Wallac Victor; Protocol: Luciassay 10sec). 24 hours thereafter, the two remaining plates (the other 0 Gy and the 6 Gy one) were treated and measured likewise.

2.2.11 Transmigration assay

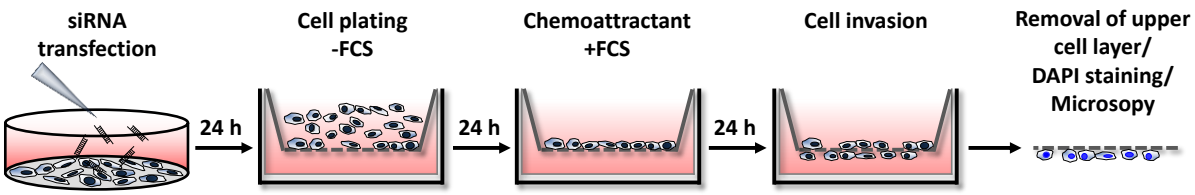


Figure 7: Methodology of transmigration assay. The procedure of a transmigration assay is hereby depicted along with the respective the time scale.

For transmigration assays (Figure 7), knockdown cell cultures were washed with PBS, trypsinized, counted and single cells were plated in a BD BioCoat™ Matrigel™ Invasion Chamber under serum-free conditions (2.5 x 10^4 cells). After 24 h of cell attachment, serum-containing medium used as a chemoattractant was added into the lower buffer chamber and the cells were further incubated at 37 °C/5% CO_2 for 24 h. For analysis of transmigrated cells, the medium was removed from all wells and the inserts were cleaned from the inside with a cotton applicator in order to remove matrigel and non-invaded cells. The inserts were then transferred to another 24-well plate with 750 µl of 70% EtOH in each well for fixation, and were left there for 20 minutes. The inserts were then transferred to another 24-well plate with 750 µl PBS in each well for washing. Subsequently, the membranes of the inserts were removed using a scalpel and soaked in 50 µl of 300 ng/ml DAPI solution for 20 minutes at 37°C (in the dark). Subsequently, the cells were placed on microscopic slides (cells-side on top) followed by mounting medium and the transmigrated cells were microscopically counted.

2.2.12 Data analysis

Means ± standard deviation (SD) were calculated with reference to controls defined in a 1.0 scale. To test statistical significance, the two-sided unpaired Student´s *t*-test was applied using EXCEL® Microsoft software. Results were considered statistically significant if a *p*-value of less than 0.05 was reached.

3. Results

3.1 The impact of Survivin deletion mutants on cellular radiation response

To analyse the impact of Survivin BIR domain as well as XIAP, Microtubules and Hsp90 binding sites on three-dimensional radiation clonogenic survival, cell cycle distribution, radiation induced DNA double-strand break repair, apoptosis and transmigration, several Survivin constructs were established expressing the wild type protein [Surv. wt, comprised of amino acid residues amino acids (AA) 1-142], XIAP (ΔXIAP; AA 1-14 and 39-142), Microtubules (ΔMicTub; AA 1-98) or Hsp90 (ΔHsp90; AA 1-78 and 88-142) binding sites deletion mutants or a BIR domain deletion mutant (ΔBIR; AA 1-14 and 88-142) fused in-frame to EGFP at the carboxyterminus of the recombinant proteins (Figure 8). Subsequently, SW480 colorectal cancer cells were stably transfected and expression of Surv. wt and deletion mutants was confirmed by western blotting and fluorescence microscopy (Figure 9). pEGFP-N1 empty vector (EGFP) served as a control.

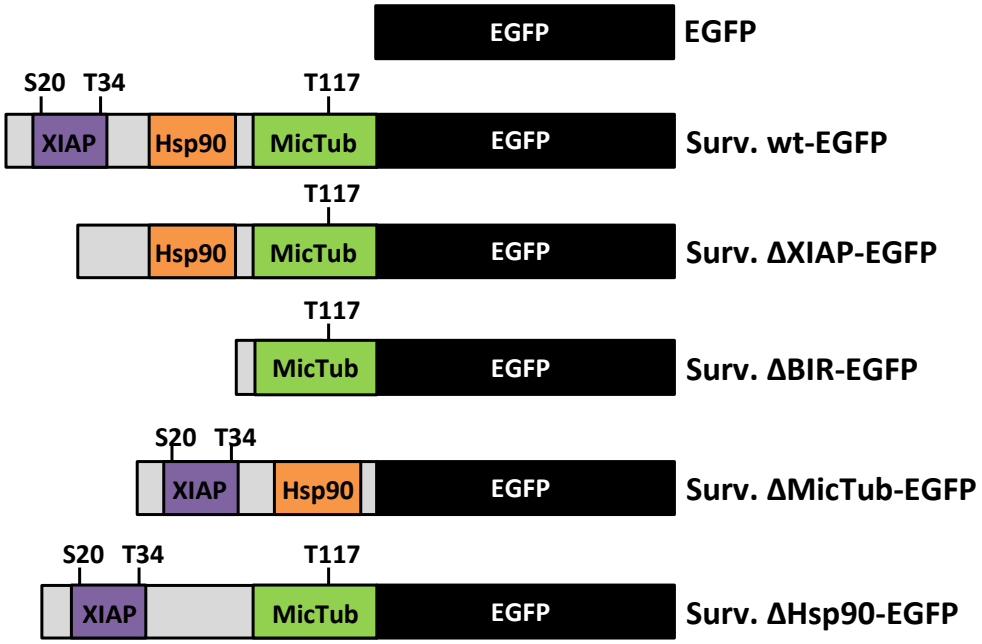
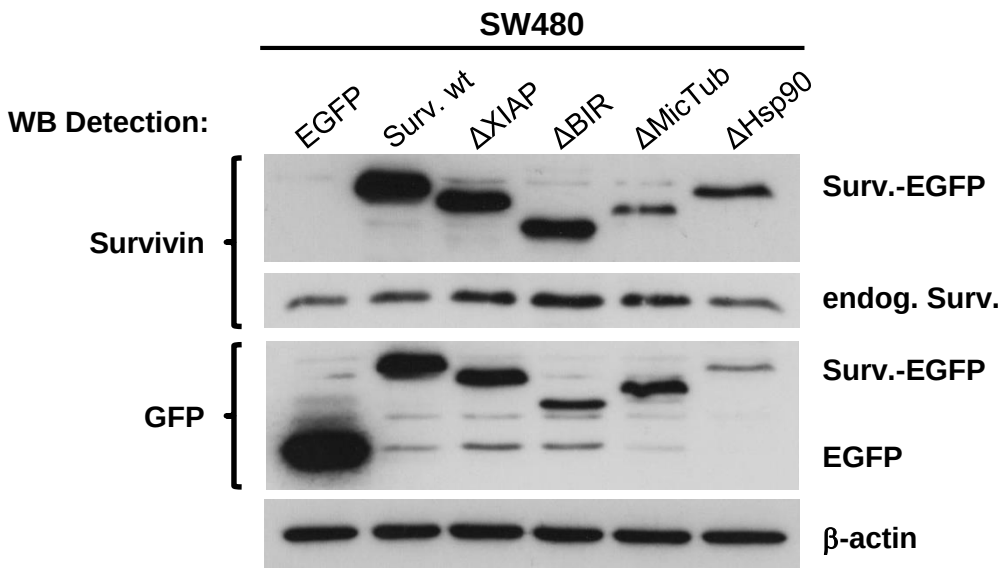


Figure 8: Symbolic diagram of Survivin-EGFP fusion constructs. Schematical representation of enhanced green fluorescent protein (EGFP), Survivin wild type-EGFP fusion protein (Surv. wt), Survivin XIAP binding site deletion mutant fused to EGFP (ΔXIAP), Survivin baculovirus inhibitor of apoptosis protein (IAP) repeat (BIR) domain deletion mutant fused to EGFP (ΔBIR), Survivin Microtubules binding site deletion mutant fused to EGFP (ΔMicTub), Survivin heat shock protein (Hsp)90 binding site deletion mutant fused to EGFP (ΔHsp90).

a)



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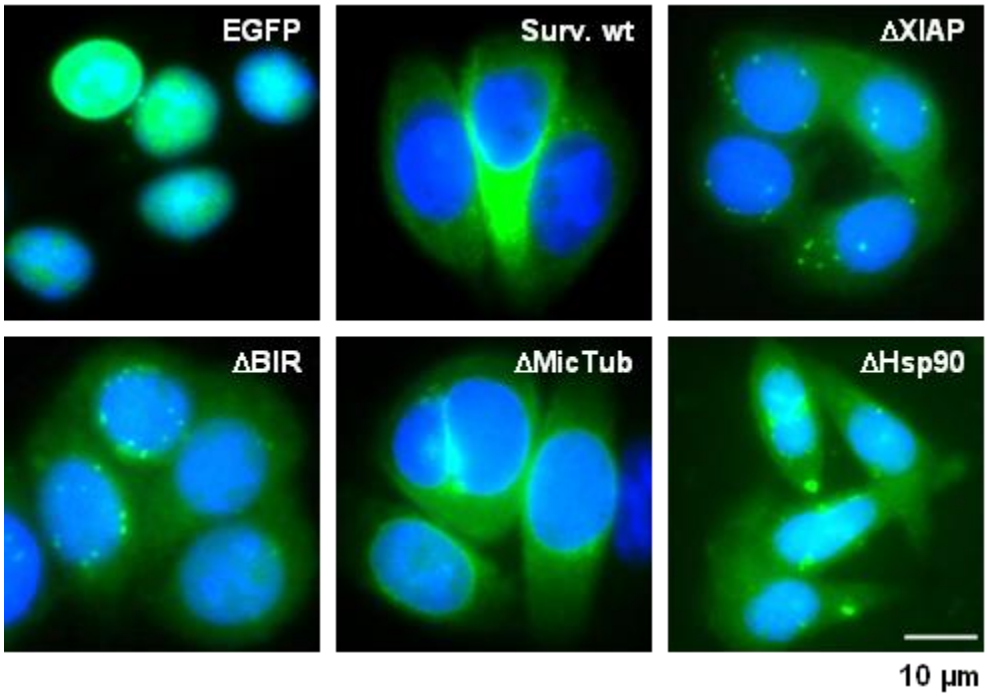


Figure 9: Stable expression of Survivin deletion mutants in SW480 cells. (a) Western blot confirmation of expression of EGFP, Surv. wt, Surv. ΔXIAP, Surv. ΔBIR, Surv. ΔMicTub, Surv. ΔHsp90 all of them fused to EGFP. Survivin and GFP antibodies were used to detect recombinant protein expression, β-actin served as loading control. (b) Fluorescence images of SW480 cells stably transfected with Survivin-EGFP expression constructs (green). Nuclei were stained with DAPI (blue). Bar: 10 µm.

3.1.1 Survivin BIR, XIAP, Microtubules and Hsp90 deletion mutants failed to reconstitute irradiation induced cell cycle arrest and apoptosis after knockdown of endogenous Survivin

SW480 cells stably overexpressing Surv. wt and Survivin deletion constructs were subjected to siRNA-mediated knockdown of endogenous Survivin without affecting expression of recombinant Survivin-EGFP fusion proteins (Figure 10).

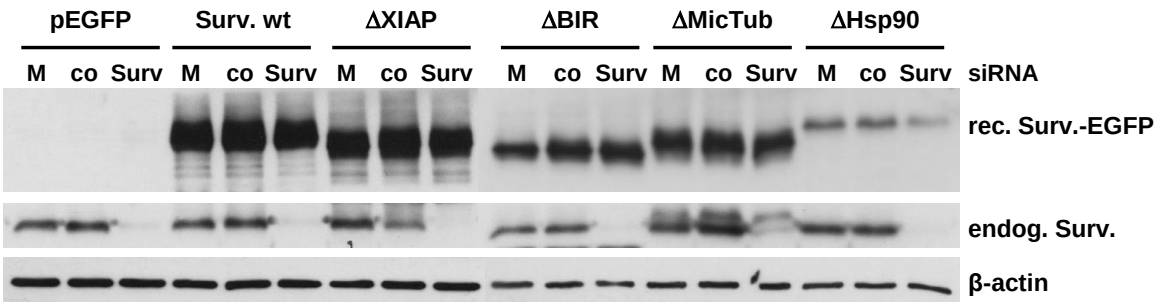


Figure 10: Western blot analysis with cells overexpressing Survivin deletion mutants. Western blot confirmation of the expression of recombinant Survivin (the indicated Survivin-EGFP fusion constructs stably expressed by SW480 cells) along with confirmation of the siRNA-mediated knockdown of endogenous Survivin. Survivin and GFP antibodies were used to detect endogenous and recombinant protein expression, β-actin served as a loading control. M: Mock-treated cells; co: Control siRNA-treated cells; Surv: cells treated with siRNA targeted against endogenous Survivin

Former studies have shown that Survivin attenuation (via siRNA) in colorectal cancer cells yielded an increased G2/M fraction of the cell cycle distribution 24 hours after knockdown, arresting the cells in a more radioresponsive phase of the cell cycle [127, 163]. In the present investigation, cell cycle analysis via flow cytometry performed with SW480 cells stably overexpressing the recombinant Survivin-EGFP fusion constructs confirmed a G2/M arrest and an increased SubG1 fraction upon knockdown of endogenous Survivin in EGFP control, Survivin ΔXIAP, ΔBIR, ΔMicTub and ΔHsp90 mutant transfected cells as compared to mock or control siRNA treated cells (Figure 11, 12a).

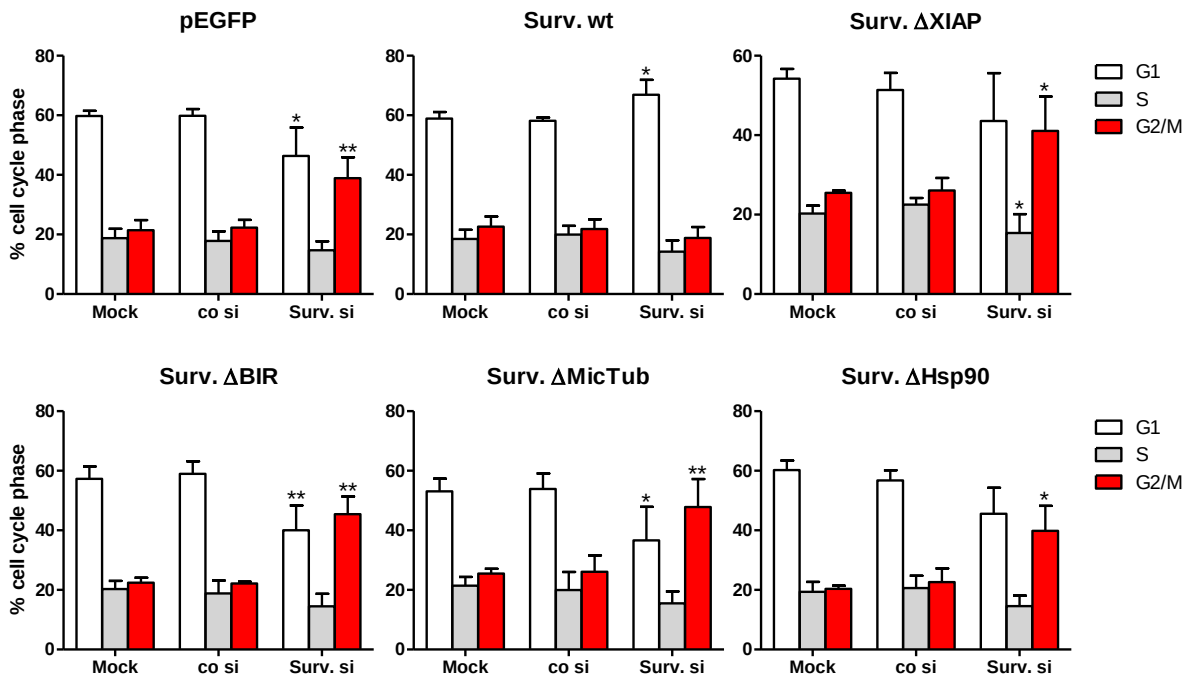
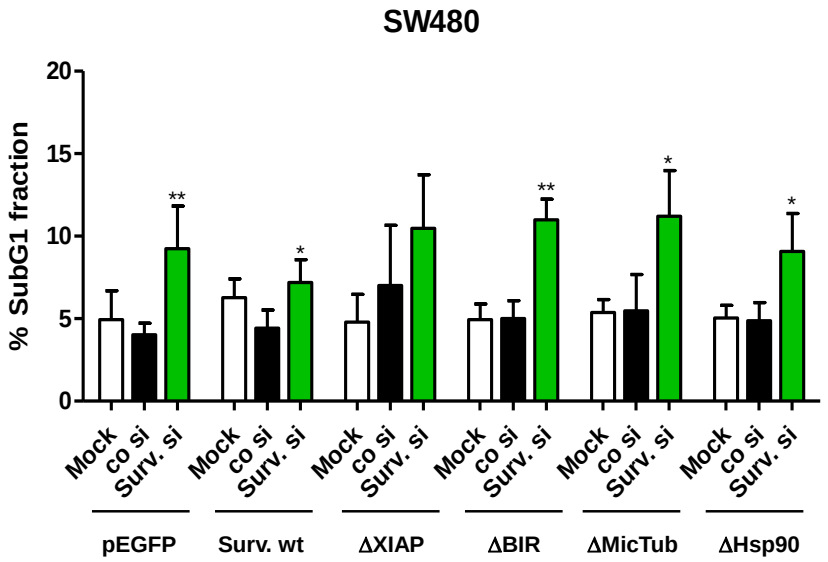


Figure 11: Cell cycle analysis of SW480 cells overexpressing Survivin deletion mutants. Cell cycle analysis using SW480 cells stably expressing Surv. ΔXIAP, Surv. ΔBIR, Surv. ΔMicTub, Surv. ΔHsp90, Surv. wt and the empty vector (for control) pEGFP. The experiments were conducted following siRNA-mediated knockdown of endogenous Survivin (Surv. si), along with control siRNA (co si) treated and mock treated (only transfection agent) cells that were used for controls. n=4; *P<0.05; **P<0.01

By contrast, stable overexpression of recombinant Survivin wt rescued SW480 cells from the G2/M arrest induced by knockdown of endogenous Survivin.

a)



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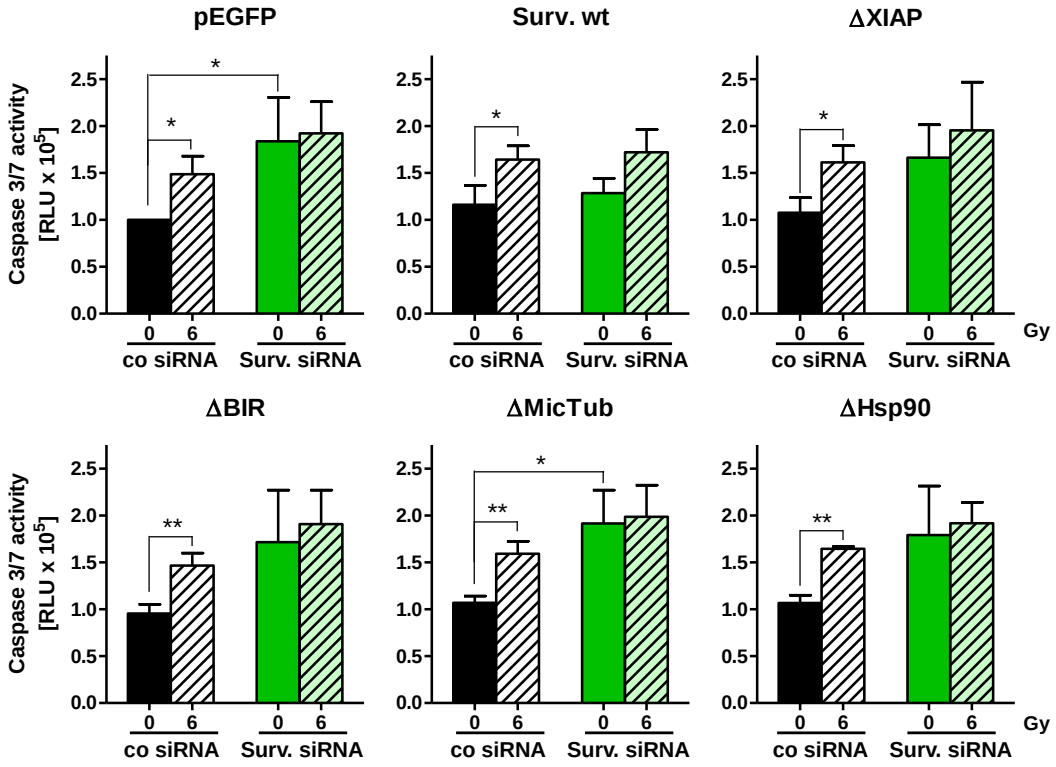


Figure 12: Sub G1 fraction and caspase 3/7 activity assays of cells overexpressing Survivin deletion mutants. (a) Sub G1 fraction via flow cytometry using SW480 cells stably overexpressing Survivin-EGFP fusion constructs after attenuation of endogenous Survivin (via siRNA). Mock and control siRNA-treated cells were used as control. (b) Caspase 3/7 activity assay performed with SW480 cells stably transfected with Survivin-EGFP fusion constructs following knockdown of endogenous Survivin (via siRNA) and irradiation with 6Gy. Control siRNA-treated cells served as a control. n=4; *P<0.05; **P<0.01

Survivin promotes tumour cell survival in part by inhibiting caspases as it has been shown by previous studies where colorectal cancer cells following treatment with siRNA targeted against Survivin responded with an increased apoptosis and caspase 3/7 activity [59, 127]. In the present study, an elevated caspase 3/7 activity was observed upon knockdown of endogenous Survivin in the deletion mutant transfected cells in comparison with control siRNA treated cells (Figure 12b). By contrast, knockdown of endogenous Survivin did not alter G2/M phase (Figure 11), SubG1 fraction or caspase 3/7 activity in Surv. wt overexpressing cells (Figure 12). Additionally, knockdown of endogenous Survivin in combination with 6 Gy irradiation did not further increase caspase 3/7 activity as compared to control siRNA treated cells (Figure 12b).

According to these results, the BIR domain as well as the XIAP, mictrotubules and Hsp90 binding sites of Survivin are important for both cell cycle regulation and apoptosis inhibition.

3.1.2 Survivin BIR domain and XIAP binding site are essential for three-dimensional radiation clonogenic survival

Previous studies conducted using colorectal carcinoma cells showed a decreased clonogenic survival following knockdown of Survivin in combination with ionizing radiation [125, 127]. Plating efficiency of non-irradiated 3D cultured cells was significantly diminished after Survivin knockdown in EGFP control, Survivin ΔXIAP, ΔBIR, ΔMicTub and ΔHsp90 expressing cells, while recombinant overexpression of Surv. wt rescued the 3D plating efficiency (Figure 13a). Overexpression of Survivin wt as well as ΔMicTub and ΔHsp90 deletion mutants rescued 3D clonogenic radiation survival while deletion of the Survivin BIR domain or XIAP binding site resulted in a significant radiosensitization upon knockdown of endogenous Survivin (Figure 13b). A summary of radiation response variables is listed in Table 5 that can be found in the appendix.

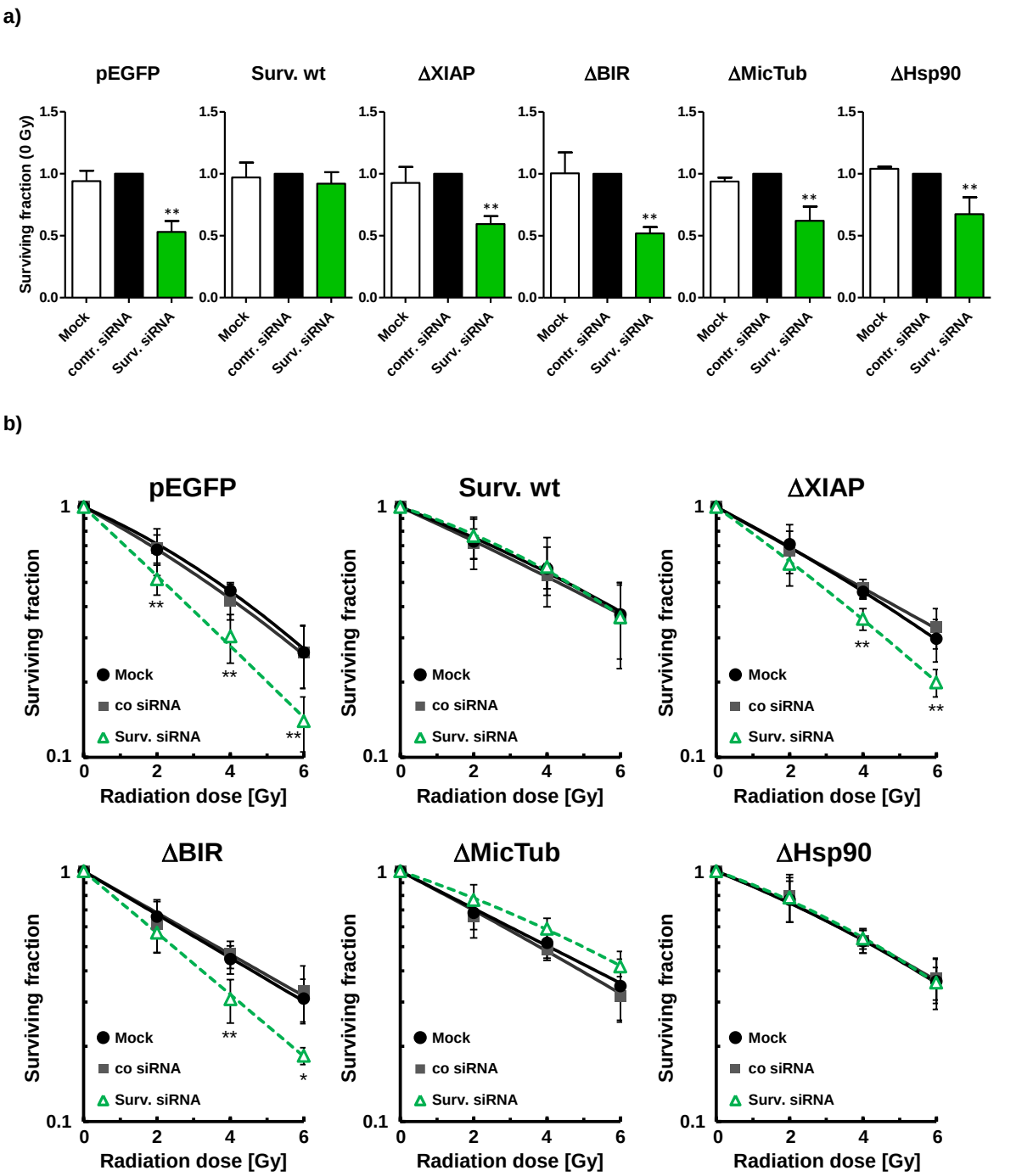


Figure 13: 3D clonogenic radiation survival of cells overexpressing Survivin deletion mutants. (a) 3D plating efficiency of cells stably overexpressing the indicated Survivin-EGFP fusion constructs after siRNA-mediated knockdown of endogenous Survivin. (b) 3D clonogenic radiation survival of cells overexpressing Survivin-EGFP fusion constructs. Cells transfected with the empty vector pEGFP served as a control. The assay commenced by plating single cells from each cell line and irradiating (X-rays) 24 hours later with the indicated doses. Colonies greater than 50 cells were counted 7 days after plating and survival curves with survival fractions normalized to the plating efficiency were fitted according to the linear quadratic equation to determine survival parameters α [Gy^{-1}] and β [Gy^{-2}]. Mock: Mock-treated cells; co siRNA: Control siRNA-treated cells; Surv. siRNA: Cells treated with siRNA targeted against endogenous Survivin; n≥4; *P<0.05; **P<0.01

According to these results, the XIAP binding site of Survivin seems to be indispensable for three-dimensional radiation clonogenic survival of SW480 colorectal cancer cells.

In order to confirm these results and ensure that the observed effect was not due to any specific behaviour of the specific SW480 clones used in these experiments, the 3D clonogenic survival assays were repeated using a set of alternative SW480 clones stably transfected with the pEGFP control vector, recombinant Survivin wild type and Survivin ΔXIAP. The radiosensitization effect which was observed in the first place in the case of the pEGFP vector control and Survivin ΔXIAP (Figure 13b) was similar with that of the alternative pEGFP #2, Surv. ΔXIAP #2 while the alternative Surv. wt #2 once again rescued the radiation clonogenic survival following knockdown of the endogenous Survivin (Figure 14).

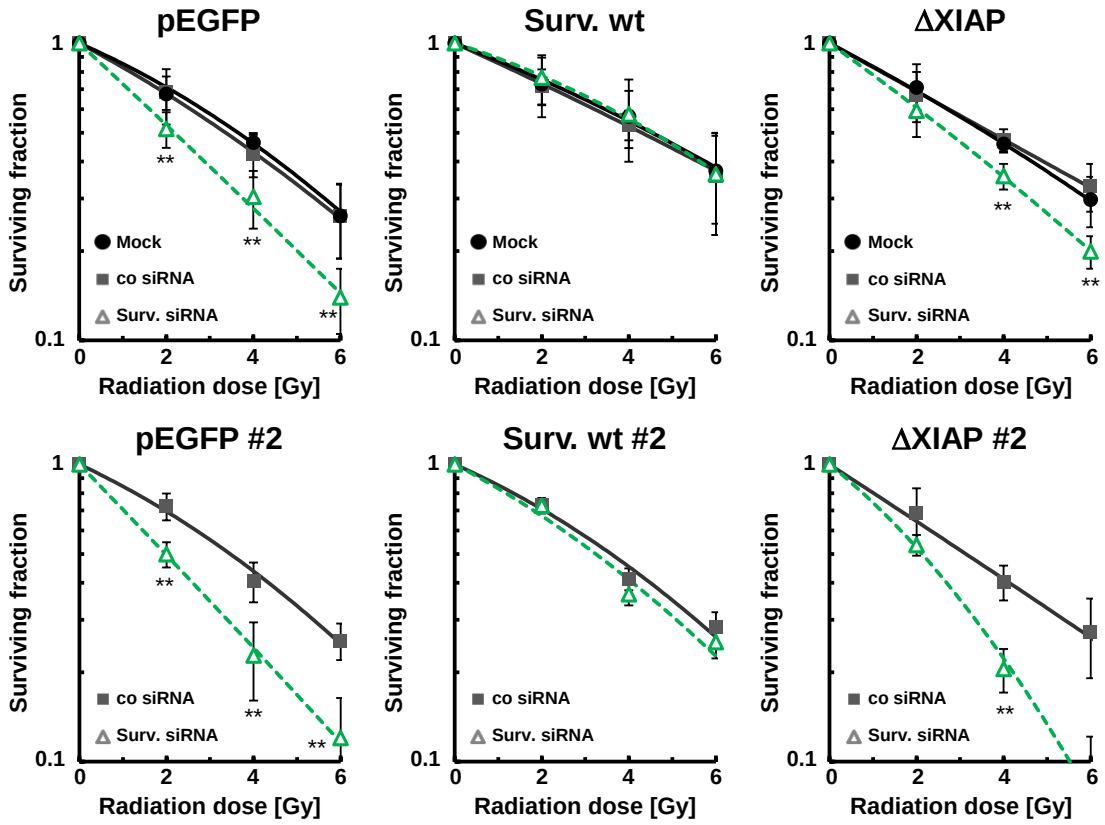


Figure 14: 3D radiation clonogenic survival assays with cells expressing alternative clones of Survivin deletion mutants. 3D clonogenic radiation survival of three alternative SW480 clones, expressing the indicated Survivin-EGFP fusion constructs. Cells transfected with the empty vector pEGFP served as a control. The colony forming assay commenced by plating single cells from each cell line (24 hours after the endogenous Survivin attenuation) and irradiating (X-rays) 24 hours later with the indicated doses. Colonies greater than 50 cells were counted 7 days after plating. Mock: Mock-treated cells; co siRNA: Control siRNA-treated cells; Surv. siRNA: Cells treated with siRNA specific for endogenous Survivin. n≥4; *P<0.05; **P<0.01

These results confirmed the importance of the XIAP binding site of Survivin for 3D radiation clonogenic survival of SW480 colorectal cancer cells.

3.1.3 Survivin BIR domain and XIAP binding site are essential for regulation of DNA double-strand break repair

Previous work showed that siRNA-mediated knockdown of Survivin in colorectal cancer as well as glioblastoma cell lines resulted in increased levels of residual DNA double-strand breaks [98, 99]. Since the molecular basis for such an effect requires further investigation, the impact of Survivin deletion mutants on DNA DSB repair was analysed via 2D and 3D immunofluorescence staining of γH2AX and 53BP1 foci of SW480 cells stably transfected with Survivin-EGFP deletion constructs following siRNA-mediated knockdown of endogenous Survivin and irradiation with 2 Gy.

Residual DNA damage in both 2D and 3D cell cultures was significantly increased upon knockdown of endogenous Survivin in empty vector EGFP, Survivin ΔXIAP and ΔBIR expressing cells, as evaluated by counting γH2AX and 53BP1 foci 24 h after irradiation with 2 Gy (Figure 15). On the other hand, overexpression of Survivin wt, ΔMicTub and ΔHsp90 constructs rescued DNA damage repair after knockdown of endogenous Survivin in both 2D and 3D IF assays (Figure 15).

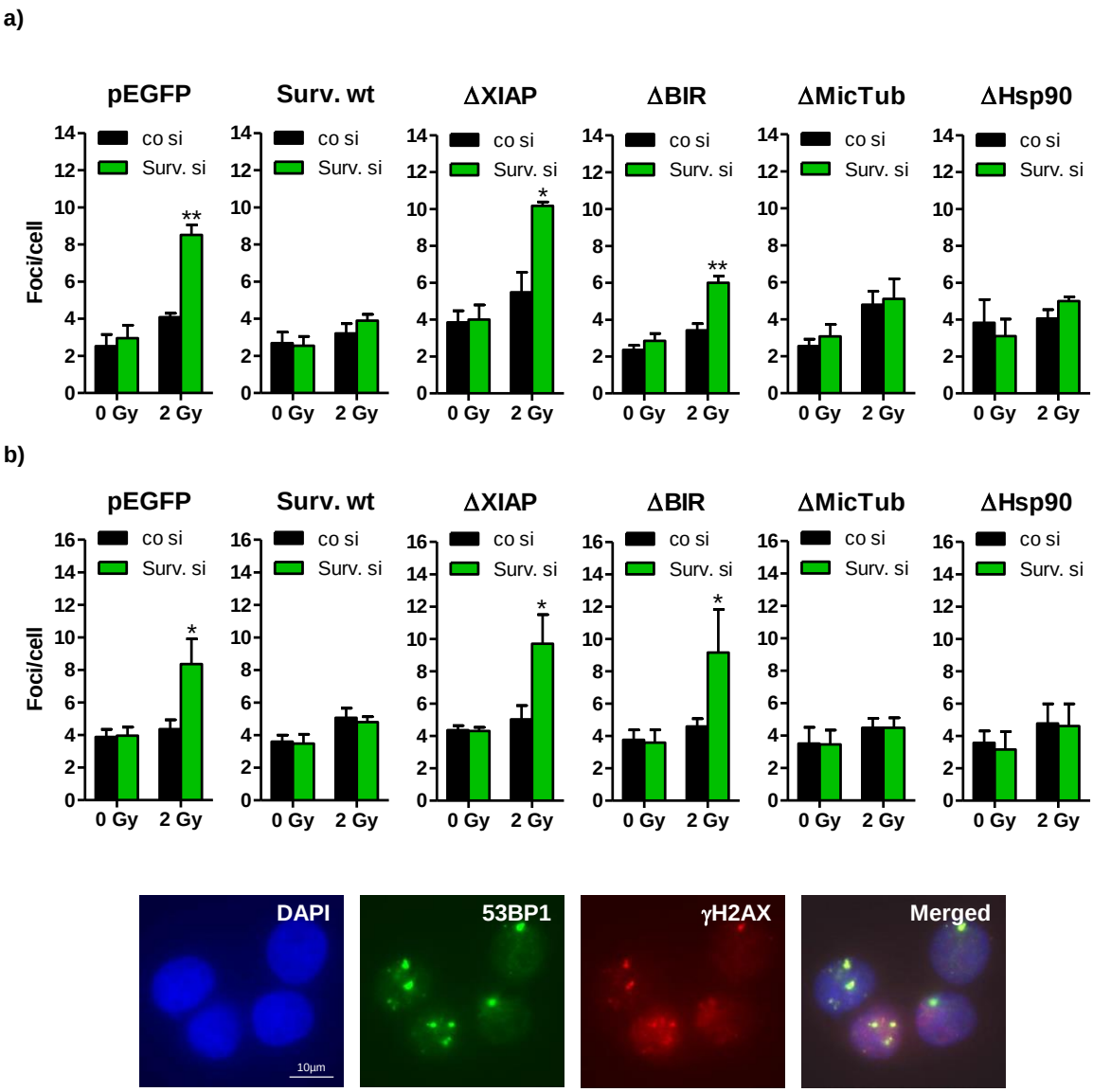


Figure 15: Immunofluorescence staining of γH2AX/53BP1 foci in cells overexpressing Survivin deletion constructs. (a) Two-dimensional and (b) three-dimensional immunofluorescence staining of γH2AX/53BP1 foci in SW480 cells overexpressing the indicated Survivin-EGFP fusion constructs. 24 hours following siRNA-mediated knockdown of endogenous Survivin, cells stably transfected with Survivn-EGFP fusion constructs were plated and 24 hours thereafter irradiated with 2Gy (0Gy served as a control). 24 hours following irradiation the cells were fixed and stained. Cells were stained for 53BP1 and γH2AX while nuclei were counterstained with DAPI (blue). Foci from at least 50 cells per condition were counted. Bar: 10µm; n=3; *P<0.05; **P<0.01

These data showed the importance of the XIAP binding site of Survivin with regard to regulation of residual DNA DSB repair.

To confirm our results, we tested alternative SW480 clones, stably transfected with pEGFP empty vector (for control), Survivin wild type and Suvivin XIAP binding site deletion construct indicated as clones #2. As a result, cells overexpressing the alternative Surv. ΔXIAP #2 along with the ones bearing the alternative empty vector pEGFP #2 showed the same significant increase of residual DNA DSBs following attenuation of endogenous Survivin and irradiation with 2Gy, whereas the Surv. wt #2 fully rescued the DNA repair (Figure 16).

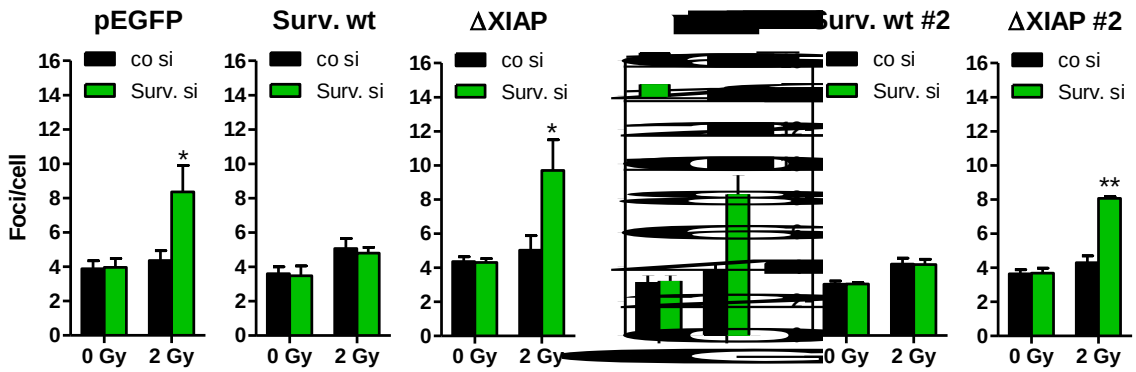


Figure 16: 3D Immunofluorescence staining of γH2AX/53BP1 foci in cells overexpressing alternative clones of Survivin deletion constructs. 24 hours following siRNA-mediated knockdown of endogenous Survivin cells stably transfected with the indicated Survivn-EGFP fusion constructs were plated and 24 hours thereafter irradiated with 2Gy (0Gy served as a control). 24 hours following irradiation the cells were fixed and stained. Cells were stained for γH2AX and 53BP1 while nuclei were counterstained with DAPI (blue). Foci from at least 50 cells per condition were counted. n=3; *P<0.05; **P<0.01

In accordance with these results, the XIAP binding site of Survivin is confirmed to be indeed essential for the regulation of DNA DSBs repair.

3.1.4 The XIAP binding site of Survivin is essential for interaction with DNA-PKcs

It has recently been shown that Survivin participates in DNA repair as a member of the non-homologous end joining (NHEJ) DNA repair machinery co-operating with factors such as DNA-PKcs [98, 99]. Thus, after having observed the importance of the XIAP binding site of Survivin in DNA DSB repair (Figures 15-16) the Survivin ΔXIAP mutant was chosen for further analysis of the interaction between recombinant Survivin and DNA-PKcs via immunoprecipitation assays that were performed using SW480 cells stably overexpressing the pEGFP empty vector (for control), Survivin-EGFP wild type and Surv. ΔXIAP mutant construct.

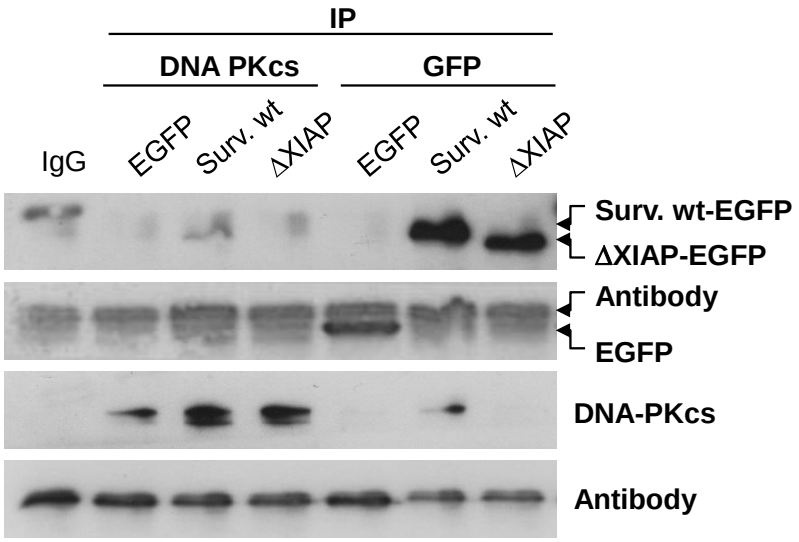


Figure 17: Immunoprecipitation experiments performed with cells overexpressing wild type Survivin and its ΔXIAP deletion mutant. Immunoprecipitation (IP) of DNA-PKcs on the left and IP of GFP on the right performed with nuclear lysates of SW480 cells stably stransfected with the pEGFP empty vector serving as a control, Survivin wild type and Surv. ΔXIAP deletion mutant after irradiation with 4Gy. Co-immunoprecipitated proteins were then detected via western blotting. Non-specific isotype antibody (immunoglobulin G or IgG) served as an additional control to the EGFP empty vector IP. Recombinant Survivin was detected using an anti-GFP antibody (due to pEGFP fusion) while DNA-PKcs was detected using an anti-DNA-PKcs antibody. Representative blot of three independent experiments (n=3).

DNA-PKcs was found to precipitate with recombinant Surv. wt (and vice versa) but not with the ΔXIAP deletion mutant indicating a possibly direct involvement of the XIAP binding site of Survivin during interaction with DNA-PKcs (Figure 17). Such an interaction can explain the significantly increased radiation-induced DNA DSBs and the clonogenic radiosensitization observed in the case of ΔXIAP mutation as opposed to Survivin wt.

3.2 The impact of Survivin phosphorylation on cellular radiation response

In order to further analyse the impact of post-translational modifications of Survivin on cellular radiation response, several phosphorylation mutants were generated involving Serine 20 (S20), Threonine 34 (T34) and Threonine 117 (T117) phosphorylation sites of the protein. In particular, those three sites were mutated to Alanine (A) to constitute a non-phosphorylatable form of the protein and Aspartic acid (D) for a phosphomimetic (Figure 18). All mutations were confirmed twice, once prior to stable transfections (via sequencing of DNA from plasmid mini preparations) and once after the stable transfections of SW480 cells via sequencing of isolated genomic DNA from the SW480 clones.

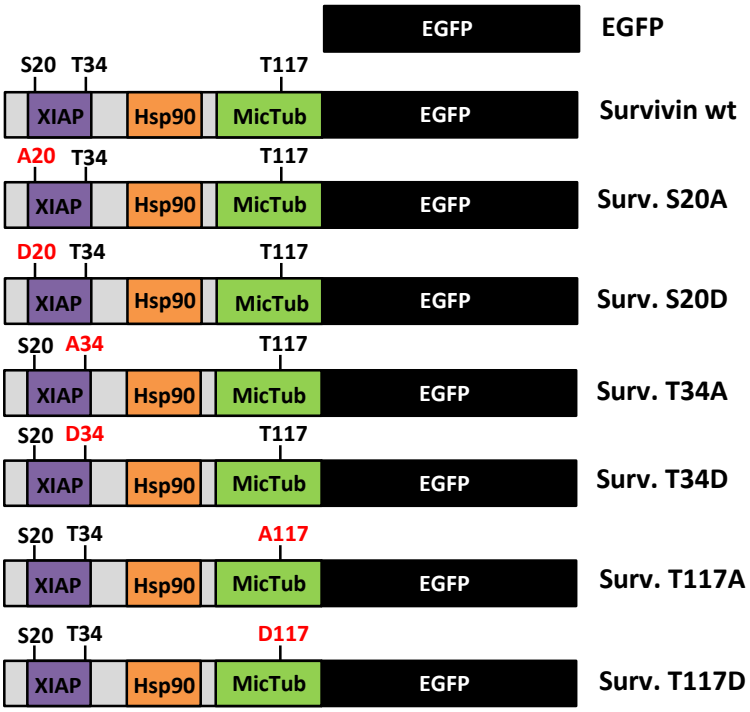


Figure 18: Symbolic representation of Survivin phosphorylation mutants. Survivin phosphorylation mutants fused with EGFP along with the recombinant wild type of the protein and the EGFP empty vector control that were used for stable transfection of SW480 cells.

3.2.1 Phosphorylation of Survivin on T34 is important for 3D radiation clonogenic survival

SW480 cells, stably expressing Survivin wt and its phosphorylation mutants were subjected to siRNA-mediated knockdown of endogenous Survivin. Prior to the 3D radiation clonogenic survival assays, the expression of the mutants as well as the knockdown of the endogenous protein were confirmed via western blotting (Figure 19).

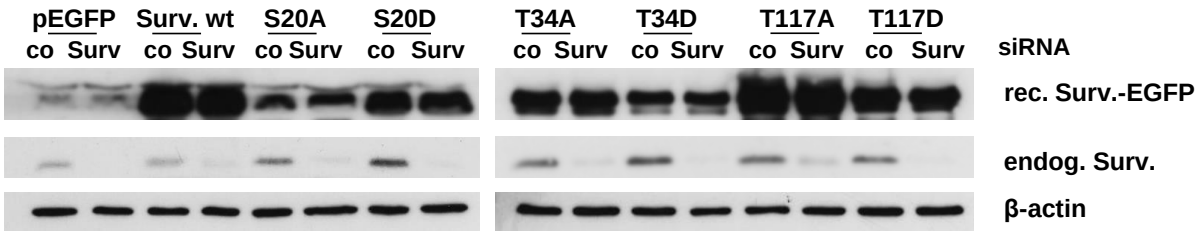


Figure 19: Western blot conducted with cells overexpressing Survivin phosphorylation mutants. Western blot confirmation of recombinant Survivin expression (Survivin-EGFP fusion constructs stably expressed in SW480 cells) along with confirmation of the siRNA-mediated knockdown of endogenous Survivin. Anti-Survivin and anti-GFP antibodies were used to detect endogenous and recombinant protein expression while β-actin served as a loading control. Co: Control siRNA-treated cells; surv: Cells treated with siRNA targeted against endogenous Survivin; representative blot of three independent experiments (n=3).

Plating efficiency of non-irradiated 3D cultured cells was significantly compromised after attenuation of endogenous Survivin in EGFP control, Survivin S20A, S20D, T34A, T34D, T117A and T117D expressing cells, while recombinant overexpression of Surv. wt rescued the 3D plating efficiency (Figure 20a). On the contrary, overexpression of Surv. wt as well as S20D, T34D, T117A and T117D phosphorylation mutants rescued 3D clonogenic radiation survival while the non-phosphorylatable form of Survivin S20A and especially the T34A resulted in a radiosensitization effect even prior to the knockdown of endogenous Survivin, thus behaving in a dominant negative manner (Figure 20b).

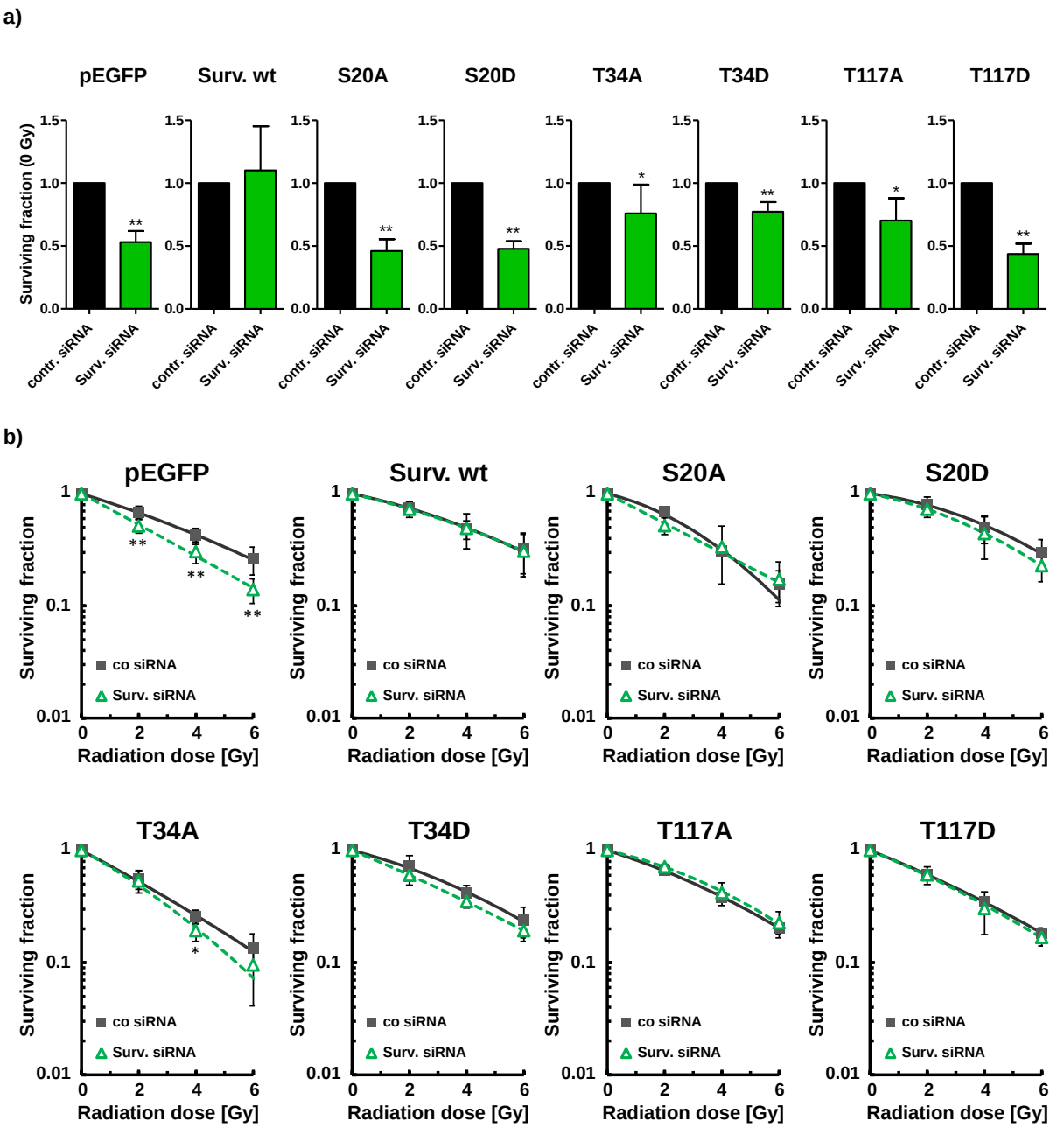


Figure 20: 3D clonogenic radiation survival of cells overexpressing Survivin phosphorylation mutants. (a) Plating efficiency of 3D clonogenic radiation survival assay performed with SW480 cells that overexpress Survivin-EGFP phosphorylation mutants following siRNA-mediated attenuation of endogenous Survivin. (b) 3D radiation clonogenic survival of cells overexpressing the indicated Survivin-EGFP phosphorylation mutants. Cells transfected with the empty vector pEGFP served as a control. The colony forming assays commenced by plating single cells from each cell line and irradiating (X-rays) 24 hours later with the indicated doses. Colonies greater than 50 cells were counted 7 days following plating. n≥3; *P<0.05; **P<0.01

A summary of radiation response variables is listed in Table 6 that can be found in the appendix.

Here it is shown that phosphorylation of Survivin on S20 and T34 is essential for clonogenic radiation survival (Figure 20b). Both phosphorylation sites are located within the XIAP binding site of Survivin (Figure 18). However, due to the poor viability of the S20A mutation, the T34A mutant was chosen for further analysis as well as comparison with the ΔXIAP construct. Thus, prior to the direct comparison of those two mutations (ΔXIAP and T34A) the behaviour of the T34A mutant in 3D radiation clonogenic survival assays was confirmed using alternative SW480 clones stably overexpressing phospho-mutant constructs (Figure 21) in additional clonogenic survival assays. The alternative clone expressing the T34A mutation named T34A #2 revealed comparable radiosensitization as shown for the first clone (T34A), while the phosphomimetic Survivin T34D clone #2 (T34D #2) dispayed similar radioresistance as the first clone (T34D) (Figure 21).

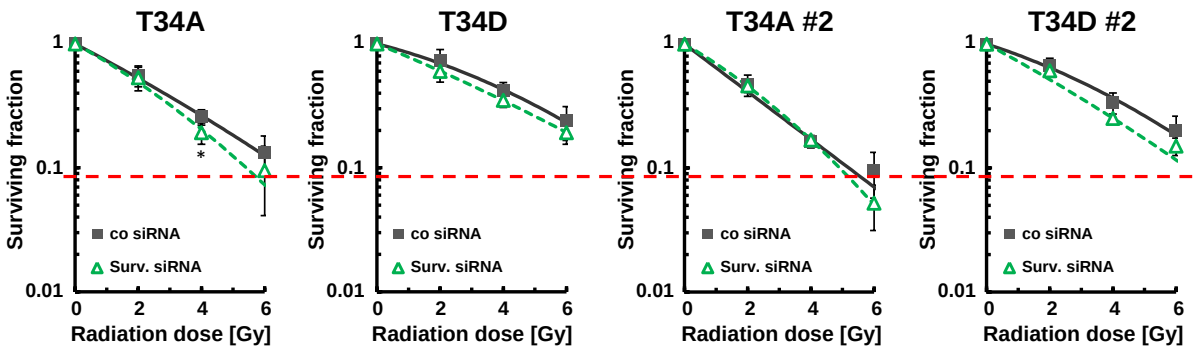


Figure 21: 3D clonogenic radiation survival of cells overexpressing alternative clones of Survivin phospho-mutants. 3D clonogenic radiation survival assays conducted with SW480 cells stably expressing the non-phosphorylatable form of Survivin T34A and the phosphomimetic T34D mutants along with the respective alternative clones (marked with #2) following siRNA-mediated knockdown of the endogenous Survivin. The assay commenced by plating single cells from each cell line and irradiating (X-rays) with the indicated doses. Colonies greater than 50 cells were counted 7 days after plating. n≥3; *P<0.05; **P<0.01

To compare the impact of Survivin T34 phosporylation with the XIAP binding site with regard to radiation survival, SW480 cells stably transfected wtth Surv. wt, Surv. ΔXIAP, Surv. T34A and Surv. T34D were subjected to siRNA-mediated knockdown of endogenous Survivin. Both the expression of the recombinant Survivin and the knockdown of the endogenous protein were confirmed via western blotting (Figure 22).

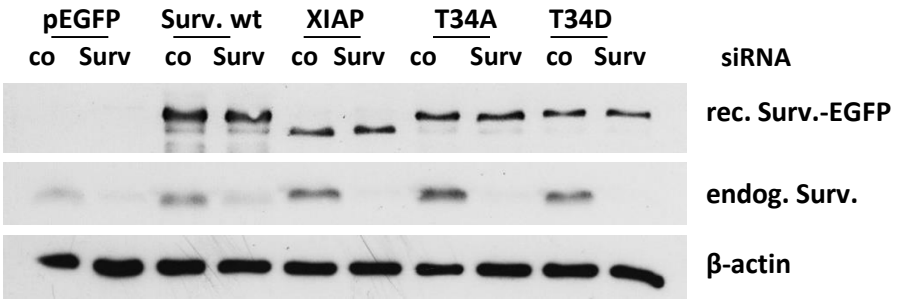
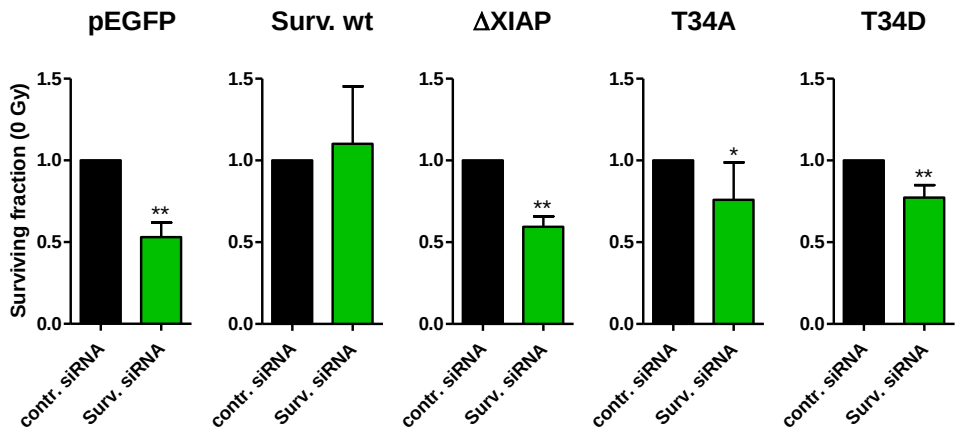


Figure 22: Western blotting performed with cells overexpressing Survivin ΔXIAP deletion mutant and Survivin T34A or T34D phospho-mutant. Western blot confirmation of the expression of recombinant Survivin along with confirmation of the siRNA-mediated knockdown of endogenous Survivin. Anti-Survivin and anti-GFP antibodies were used to detect endogenous and recombinant protein expression while β-actin served as a loading control. Co: Control siRNA-treated cells; Surv: Cells treated with siRNA targeted against endogenous Survivin. Representative blot of four independent experiments (n=4).

Plating efficiency of non-irradiated 3D cultured cells was significantly diminished after Survivin knockdown in EGFP control, Survivin ΔXIAP, T34A, and T34D expressing cells while overexpression of Survivin wt rescued the 3D plating efficiency (Figure 23a). However, overexpression of Survivin wt as well as T34D phospho-mutants rescued 3D clonogenic radiation survival while deletion of Survivin XIAP binding site or mutation of Threonine 34 to Alanine resulted in a radiosensitization upon knockdown of endogenous Survivin in the case of the ΔXIAP and even prior to the knockdown in the case of T34A mutation (Figure 23b).

a)



b)

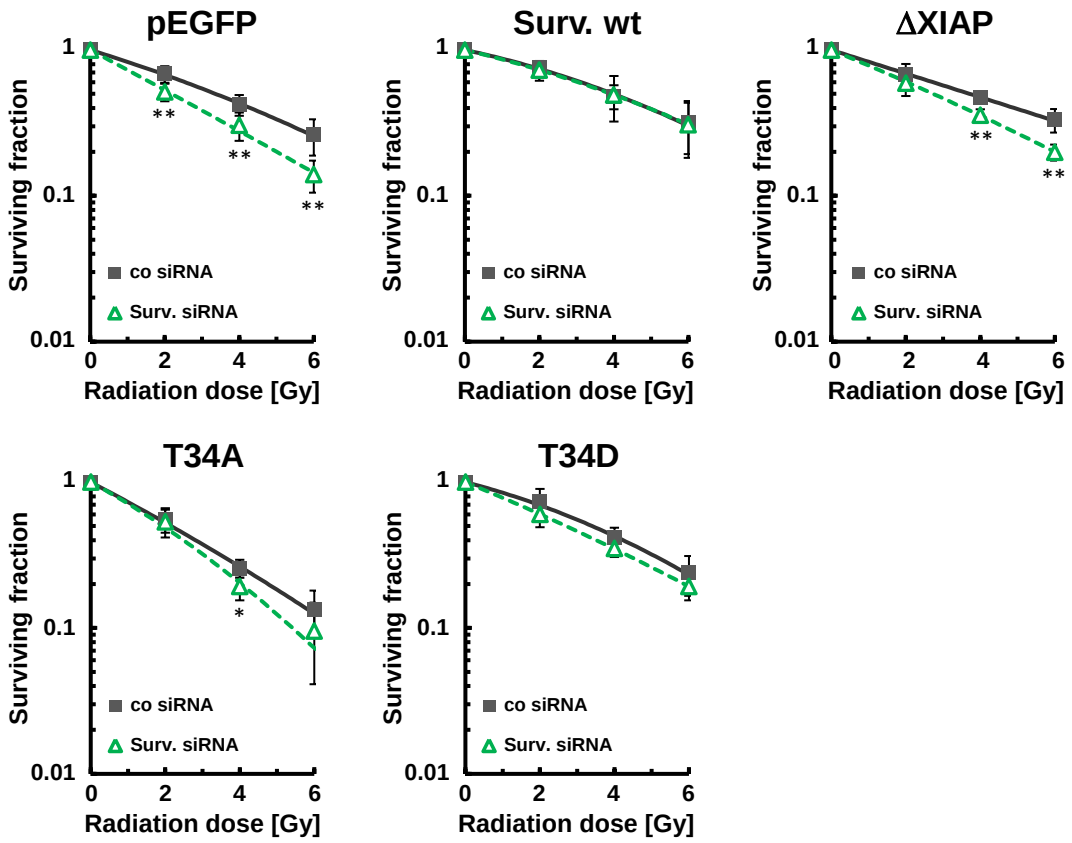


Figure 23: 3D clonogenic radiation survival of cells overexpressing the indicated Survivin mutants. (a) 3D plating efficiency of cells stably overexpressing Survivin-EGFP fusion constructs namely Surv. wt, Surv. ΔXIAP, Surv. T34A and Surv. T34D along with the empty vector pEGFP for control. (b) 3D clonogenic radiation survival of cells overexpressing Survivin-EGFP fusion constructs. The assay commenced by plating single cells from each cell line and irradiating (X-rays) 24 hours later with the indicated doses. Colonies greater than 50 cells were counted 7 days after plating. n≥4; *P<0.05; **P<0.01

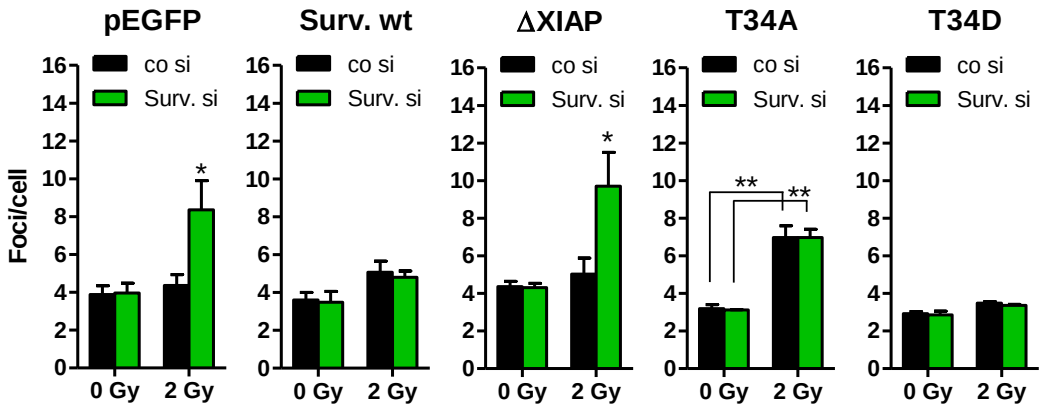
These results indicate the importance of the T34 phosporylation site of Survivin, while it is not clear whether XIAP binding or binding of different proteins to the XIAP binding site is fostered by T34 phosphorylation.

3.2.2 The XIAP binding site and the T34 phosphorylation site of Survivin are essential for regulation of residual DNA DSBs repair

Following the observation of the impact of Survivin deletion mutant ΔXIAP on DNA DSBs repair (Figure 15) and the effect of the T34A mutant on 3D radiation clonogenic survival (Figure 23b), the latter was chosen for further investigation regarding DNA damage repair along with direct comparison with the ΔXIAP mutant.

Residual DNA damage (as evaluated by counting γH2AX and 53BP1 foci in immunofluorescence staining assays) 24 h after irradiation with 2 Gy in 3D cell cultures was significantly increased upon knockdown of endogenous Survivin in EGFP and Survivin ΔXIAP, while T34A expressing cells showed a similar increase of residual DNA damage even prior to attenuation of endogenous Survivin (Figure 24a).

a)



b)

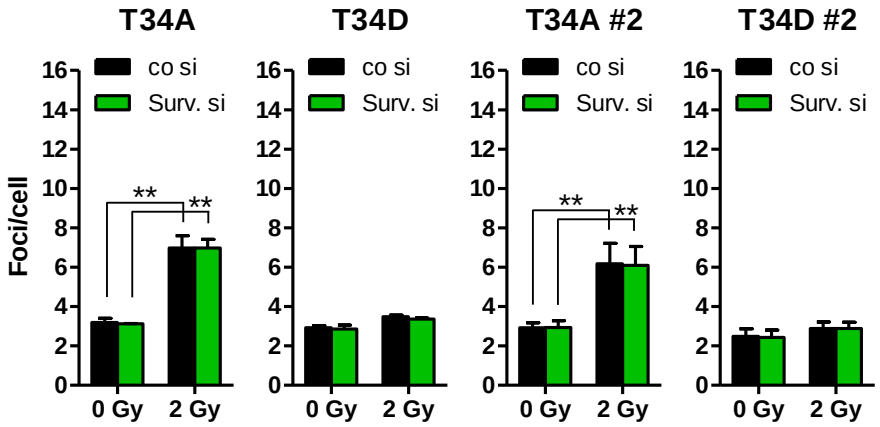


Figure 24: 3D immunofluorescence staining of γH2AX/53BP1 foci in cells overexpressing Survivin deletion and phosphorylation mutants. (a) 3D IF of γH2AX/53BP1 foci used as a marker for residual DNA DSBs. 24 hours following siRNA-mediated knockdown of endogenous Survivin cells stably transfected with the indincated Survivn-EGFP fusion constructs were plated and 24 hours thereafter irradiated with 2Gy (0Gy served as a control). 24 hours following irradiation the cells were fixed and stained. Cells were stained for 53BP1 and γH2AX while nuclei were counterstained with DAPI (blue). Foci from at least 50 cells per condition were then counted. (b) 3D immunofluorescence staining of alternative (#2) Survivin T34A and T34D clones for confirmation of the previous results. n=3; *P<0.05; **P<0.01

However, overexpression of Survivin wt and T34D constructs rescued the DNA damage repair after knockdown of endogenous Survivin (Figure 24a).

In order to confirm that these results were not clone-dependent, the 3D immunofluorescence staining assays were repeated using alternative SW480 clones expressing Survivin T34A and T34D phospho-mutants (Figure 24b). As a result, an alternative SW480 Survivin T34A #2 clone showed similar significant increase of residual DNA DSBs following irradiation with 2Gy, whereas an alternative SW480 clone expressing the phospho-mimetic Survivin T34D #2 fully rescued residual DNA DSBs repair (Figure 24b).

3.2.3 Mutation of the Survivin T34 phosphorylation site to its non-phosphorylatable form T34A prevents interaction with DNA-PKcs

The XIAP binding site as well as the T34 phosphorylation site of Survivin were shown to be implicated in the repair of residual DNA DSBs (Figure 24a). Thus, the Survivin phospho-mutant T34A along with its phosphomimetic T34D were further analysed in terms of DNA-PKcs interaction. In order to investigate such an interaction, SW480 cells stably overexpressing those Survivin mutants were subjected to subcellular fractionation following irradiation with 4 Gy and their nuclear fractions were used for GFP immunoprecipitation assays (due to the Survivin-EGFP fusion) (Figure 25).

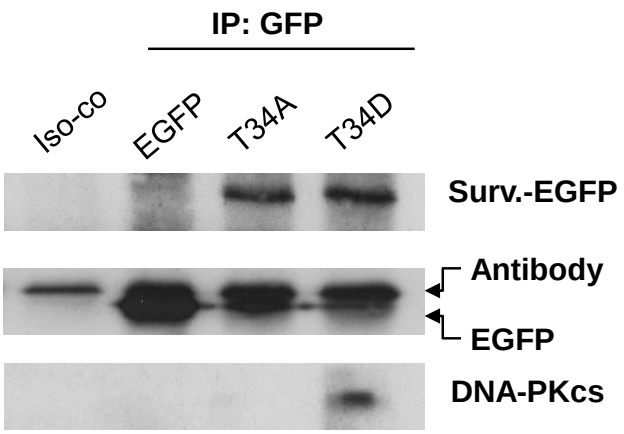


Figure 25: Immunoprecipitation experiment with cells overexpressing Survivin phospho-mutants. IP of GFP performed from nuclear lysates of SW480 cells stably stransfected with the pEGFP empty vector serving as a control, Survivin T34A and Survivin T34D after irradiation with 4Gy. Associated proteins were then detected via Western blotting. Non-specific isotype antibody (immunoglobulin G or IgG) served as an additional control to the EGFP empty vector IP. Recombinant Survivin was detected using an anti-GFP antibody while DNA-PKcs was detected using an anti-DNA-PKcs antibody. Representative blot of n=4.

The phosphomimetic form of recombinant Survivin T34D was found to co-immunoprecipitate with DNA-PKcs while the non-phosphorylatable construct of Survivin T34A did not show such an effect proving Survivin phosphorylation at Threonine 34 important for interaction with DNA-PKcs. The fact that the DNA repair factor DNA-PKcs did not co-immunoprecipitate with the Survivin T34A mutant might explain at least in part the significantly increased radiation-induced residual DNA DSBs shown in the immunofluorescence staining assays of the T34A mutant versus the phosphomimetic T34D. Additionally, this effect serves as an explanation for the clonogenic radiosensitivity observed in the case of T34A mutant as compared to Survivin wt and T34D.

3.3 Survivin BIR domain, XIAP, Microtubules and Hsp90 binding sites are essential for transmigration capability of colorectal cancer cells

It was recently reported that knockdown of Survivin and/or XIAP in many colorectal cancer cell lines leads to a significantly diminished migration capacity [164] revealing the importance of Survivin in colorectal cancer cell motility. In this work, attenuation of endogenous Survivin decreased transmigration of EGFP control, and ΔXIAP expressing cells compared to control siRNA-treated cells while ΔBIR, ΔMicTub and ΔHsp90 constructs acted as dominant negative mutants already significantly repressing transmigration of control cells (Figure 26).

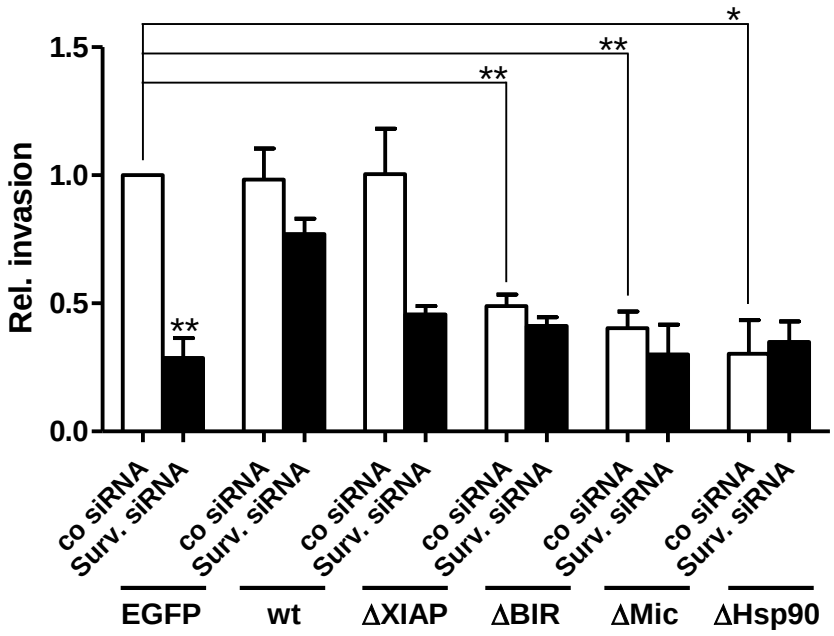


Figure 26: Transmigration assay performed with cells overexpressing Survivin deletion mutants. Transmigration assay performed with SW480 cells stably transfected with the indicated Survivin-EGFP fusion constructs. The assay commenced by plating cells in inserts containing a 8 µm pore size membrane covered with a thin layer of matrigel (a laminin-rich extracellular matrix) under serum-free conditions 24h after siRNA-mediated knockdown of endogenous Survivin followed by addition of a chemo-attractant (serum-enriched medium). The cells that went through the membrane of the inserts (transmigrated cells) were fixed and nuclei were counterstained with DAPI. co siRNA: Control siRNA-treated cells; Surv. siRNA: Cells treated with an siRNA targeted against endogenous Survivin. n=2; *P<0.05; **P<0.01

Knockdown of endogenous Survivin did not further decrease the transmigration rate of ΔBIR, ΔMicTub and ΔHsp90 stably transfected cells. It is hereby shown that not only the binding site of XIAP, but also those of Microtubules and Hsp90 are involved in the transmigration process suggesting further mechanisms by which Survivin regulates the motility of cancer cells.

4. Discussion

The majority of both solid and liquid human tumours are characterized by a dramatic overexpression of Survivin [59, 122, 148] as it has been reported in studies involving malignancies of lung [165], breast [166], colon [167], stomach [168], eosophagus [169], pancreas [170], liver [171], uterus [172], ovaries [173], Hodgkin's disease [174], non-Hodgkin's lymphoma [175, 176], leukaemias [177, 178] neuroblastoma [179, 180], phaeochromocytoma [181], soft-tissue sarcomas [182], gliomas [183] and melanoma [184]. The respective normal tissues, however, did not show an expression of Survivin. The molecular background of Survivin's overexpression in tumours is not a mere effect of mitotic indices that reflect a higher number of proliferating cells in cancers [184]. Survivin gene expression is potentially deregulated showing amplification of its locus on 17q25 in neuroblastoma [179], selective demethylation of its exon 1 in ovarian cancer but not in normal ovaries [185] as well as loss of wildtype p53 [59]. Survivin is transcriptionally repressed by wild-type p53 possibly via modifications in chromatin structure affecting promoter accessibility [145, 146, 186]. In the case of colorectal cancer, up-regulation of Survivin is thought to originate from APC mutations and aberrant stabilization of β-catenin [141].

In particular, Survivin is involved in colorectal carcinogenesis as it stimulates the transition of adenomas with low-grade dysplasia into dysplastic lesions and colonic carcinomas in situ [148, 187, 188]. Consequently, its expression in malignant tissue renders a prognostic factor due to its diagnostic relevance [148]. Increased Survivin expression was found to be associated not only with a higher risk of tumour recurrences, but also lymph node metastases as well as shorter survival in cases of non-small cell lung cancer [189], T1 bladder carcinoma [190], meningiomas [191], rectal adenocarcinoma [192] and locally advanced prostate cancer [193] treated with radiotherapy or chemo-radiation.

In addition, there is substantial evidence proving Survivin to be a radiation resistance factor [123-125] the attenuation of which leads to radiosensitization of tumour cells both *in vitro* and *in vivo* [112, 148]. Studies conducted *(in vitro)* using glioblastoma, colorectal carcinoma, hepatocellular carcinoma and NSCLC cells showed a decreased clonogenic survival following knockdown of Survivin in combination with ionizing radiation as well as reduced tumour growth in xenograft transplant models [112, 148].

Apart from participating in cell division and apoptosis, Survivin is a nodal tumour protein involved in a multitude of signaling pathways and transcriptional networks [194]. One striking feature of this protein is its pronounced ability to co-operate with a plethora of protein partners such as other members of the IAP family (like XIAP) as well as components of the DNA DSB repair machinery (such as DNA-PKcs). Thus, a further investigation of its relationship with members of the IAP family as well as other protein partners (such as Hsp90) and DNA repair proteins [98] is necessary in order to figure out the regulatory role of Survivin in terms of tumour cell survival and modulation of treatment response.

The expression of Survivin is controlled at various levels via intracellular sequestration as well as protein stability and transcription [148]. In non-malignant cells its expression is transcriptionally regulated in a cell-cycle-dependent manner reaching its peak at mitosis involving cell-cycle-dependent/cell-cycle gene homology region (CDE/CHR) elements found in its promoter [102]. On the contrary, in tumour cells its expression is controlled regardless of mitosis [195] by means of various oncogenic pathways [148]. It has been shown that an siRNA-mediated knockdown of Survivin in colorectal cancer cells leads to an increased G2/M fraction of the cell cycle distribution 24 hours following the knockdown, blocking the cells in a more radiosensitive stage of the cell cycle [127, 163]. In the course of this project, after cell cycle analysis experiments colorectal cancer cells stably transfected with Survivin lacking its BIR domain, XIAP, Microtubules or Hsp90 binding sites were shown to accumulate in the

G2/M phase after knockdown of the endogenous protein revealing an implication of these particular binding sites of the protein in cell cycle regulation.

It has been demonstrated that colorectal cancer cells following treatment with siRNA targeted against Survivin respond with increased apoptosis and caspase 3/7 activity supporting the fact that Survivin promotes tumour cell survival by inhibiting caspases [59, 127]. With the exception of XIAP, none of the IAPs is able to directly bind caspases [21]. It is thus believed that Survivin can inhibit apoptosis by interacting with other protein partners, with the Survivin-XIAP complex being a characteristic example of such an interaction during which the XIAP binding site of Survivin associates with discontinuous sites in XIAP BIR1 and BIR3 [22, 31]. In this study, it was observed that colorectal cancer cells stably expressing Survivin deletion mutants (lacking the BIR domain/the XIAP/Microtubules/Hsp90 binding site) showed an incompetence to inhibit apoptosis following an siRNA-mediated knockdown of the endogenous protein. Therefore, the BIR domain of the protein as well as the XIAP, Microtubules and Hsp90 binding sites that were abolished due to the mutations, are apparently involved in apoptosis inhibition.

The radiation clonogenic survival of colorectal cancer cells has been shown to decrease after siRNA-mediated attenuation of Survivin [98, 99, 127, 164]. In the present investigation, colorectal cancer cells transfected with recombinant Survivin lacking its BIR domain or its XIAP binding site were significantly radiosensitized while transfection of cells with Survivin ΔMicTub or ΔHsp90 deletion mutants fully rescued radiation clonogenic survival following the knockdown of the endogenous protein, demonstrating a radioresistance equal to that of cells overexpressing wild-type recombinant Survivin. Furthermore, cells expressing the non-phosphorylatable form of Survivin S20A (Serine 20 Alanine) or T34A (Threonine 34 Alanine), both of which are located within the XIAP binding site, revealed a radiosensitizing effect even prior to endogenous Survivin attenuation thus behaving like dominant negative mutants. On the other hand, cells expressing the phosphomimetic version of Survivin S20D (Serine 20

Aspartic acid) or T34D (Threonine 34 Aspartic acid) could rescue clonogenic survival. However, cells transfected with Survivin phospho-mutant T117A or T117D, located on the Microtubules binding site of the protein, did not show such a radiosensitizing effect. Consequently, either the interaction between Survivin and XIAP or the XIAP binding site per se and/or the S20 or T34 phosphorylation sites, located in that region are vital for radiation clonogenic survival of colorectal cancer cells. These results are in line with previously published data according to which a phosphomimetic mutant of Survivin namely T34E (Threonine 34 Glutamic acid) showed a higher radioresistance than the corresponding kinase-dead mutant T34A in human cervical carcinoma HeLa cells [187]. Additionally, according to research conducted in our group, depletion of Survivin and/or XIAP resulted in comparable effects regarding 3D clonongenic radiation survival [164].

Chakravarti and colleagues were the first to observe that Survivin attenuation decreases tumour cell survival upon irradiation via caspase-independent mechanisms such as DNA repair deficiency [124]. For instance, following Survivin knockdown via siRNA, ASO (anti-sense oligonucleotide) or YM155 (a small molecule inhibitor of Survivin expression) in colorectal and NSCLC cells, a hampered DNA repair was reported [126-128]. In addition, it has recently been shown that siRNA-mediated attenuation of Survivin followed by irradiation, resulted in a reduction of Ser2056 autophosphorylation of DNA-PKcs, a decrease of DNA-PKcs kinase activity and increased radiation-induced residual DNA damage in colorectal cancer and glioblastoma cells [98, 99]. In order to further investigate the implication of Survivin in DNA repair regulation, immunofluorescence evaluation of DSBs was performed with cells overexpressing mutated constructs of this protein. Thus, colorectal cancer cells transfected with recombinant Survivin following deletion of its XIAP binding site or BIR domain revealed a significantly increased residual DNA damage following ionizing radiation as compared to the overexpression of recombinant wild-type Survivin. On the contrary, cells transfected with Survivin lacking the Microtubules or Hsp90 binding site did not show such an

effect being able to substitute the function of endogenous Survivin in terms of DNA repair. Apart from cells expressing Survivin ΔXIAP or ΔBIR, cells expressing the non-phosphorylatable form T34A also exhibited a significantly increased residual DNA damage following ionizing radiation. However, cells expressing the phosphomimetic form T34D successfully rescued DNA repair following irradiation. In other words, the XIAP binding site and/or the T34 phosphorylation site of Survivin are essential for radiation induced residual DNA DSBs repair as opposed to the Microtubules or Hsp90 binding sites of the protein. Regarding the T34A mutation, these results are in line with previously published data where Survivin T34A-transfected cells showed a greater DNA double-strand breakage post-irradiation in comet assays [124].

The present data regarding the radiation clonogenic survival as well as the immunofluorescence evaluation of DNA DSBs in cells overexpressing Survivin mutants were obtained using 3D cell culture systems. It has been shown that 3D cultures reflect better the physiological conditions *in vivo* in terms of cellular radiation response [196] as cell shape and cell-to-cell contact are factors of great importance regarding responsiveness to external stress [197]. Principally, 3D microenvironment has been reported to confer tumour cell radio-resistance due to epigenetic differences in terms of chromatin condensation in 3D-grown as compared to monolayer cultures [198].

According to recent studies Survivin accumulates in the nucleus after irradiation and directly interacts with DNA repair proteins (involved in NHEJ) such as DNA-PKcs and Ku70 in order to regulate DSB repair [98, 99]. In the present study, it was via immunoprecipitation experiments observed that Survivin lacking the XIAP binding site as well as the non-phosphorylatable form T34A did not precipitate with DNA-PKcs, an effect meanwhile present in the case of cells expressing wild-type recombinant Survivin (Figure 27).

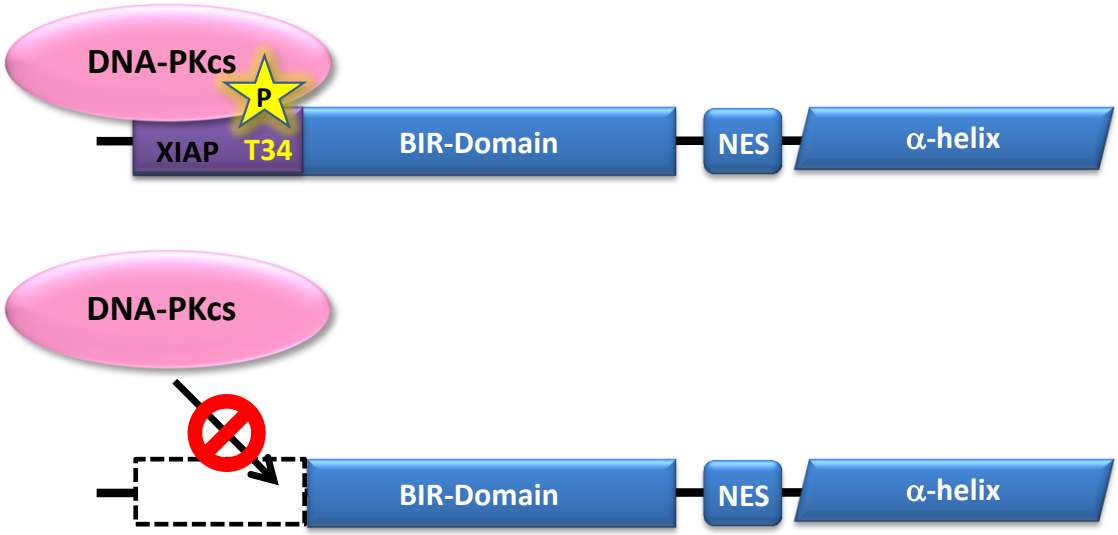


Figure 27: Symbolic diagram of Survivin wt and Surv. ΔXIAP. Representation of Survivin wild type (on top) and Survivin lacking the XIAP binding site (on the bottom). It is suggested that DNA-PKcs interacts with Survivin within its XIAP binding site and/or only when Survivin is phosphorylated at Threonine 34 while deletion of the XIAP binding site abolishes such an interaction.

Thus, it can be suggested that the XIAP binding site and/or the T34 phosporylation site of the protein are of great importance not only for apoptosis inhibition [31] but also for DNA DSB repair regulation via interaction with DNA-PKcs. The inability of those EGFP-fused constructs (Survivin ΔXIAP and Survivin T34A) to interact with this repair protein may at least in part explain both the increased residual DNA damage and the diminished radiation clonogenic survival of cells overexpressing those constructs.

Aside from its well-known functions in cellular survival regulation, apoptosis and cell cycle, Survivin has recently been described to promote migration and invasion of different tumour cells including human LoVo colorectal cancer, breast adenocarcinoma MDA-MB-231, prostate adenocarcinoma PC3, melanoma cells or melanocytes [58, 199, 200]. It has further been shown that knockdown of Survivin and/or XIAP in multiple colorectal cancer cell lines leads to a significantly diminished migration capacity [164]. Principally, the Survivin-XIAP complex protects XIAP stability from ubiquitin-dependent degradation while increases

caspase inhibition [31, 114], enhances tumour growth *in vivo* [114] and participates in NF-κB activation [58]. NF-κB activation leads to a transcriptional up-regulation of Survivin [201] resulting in an amplification loop that favours cell survival [202] specifically in tumours where elevated NF-κB activity is related to aggressive disease and metastasis [58, 203]. In more detail, the Survivin-XIAP complex activates NF-κB, triggering NF-κB-dependent transcription of fibronectin, an extracellular matrix protein [58] that in turn engages β1 integrins at cell surface, activating cell motility kinases, Src and focal adhesion kinase (FAK) resulting in tumour cell migration, invasion and metastatic dissemination *in vivo* [58]. Despite the importance of the Survivin-XIAP complex, modulating tumour cell motility factors such as fibronectin expression and FAK, it is hereby shown that not only the XIAP binding site of Survivin but also those of Microtubules and Hsp90 are involved in cell migration, suggesting further mechanisms via which Survivin participates in cancer cell motility.

In brief, these results confirm Survivin to function as a radiation resistance factor modulating cellular radiation response via DNA DSB repair, induction of cell cycle arrest, apoptosis as well as cell motility (Figure 28).

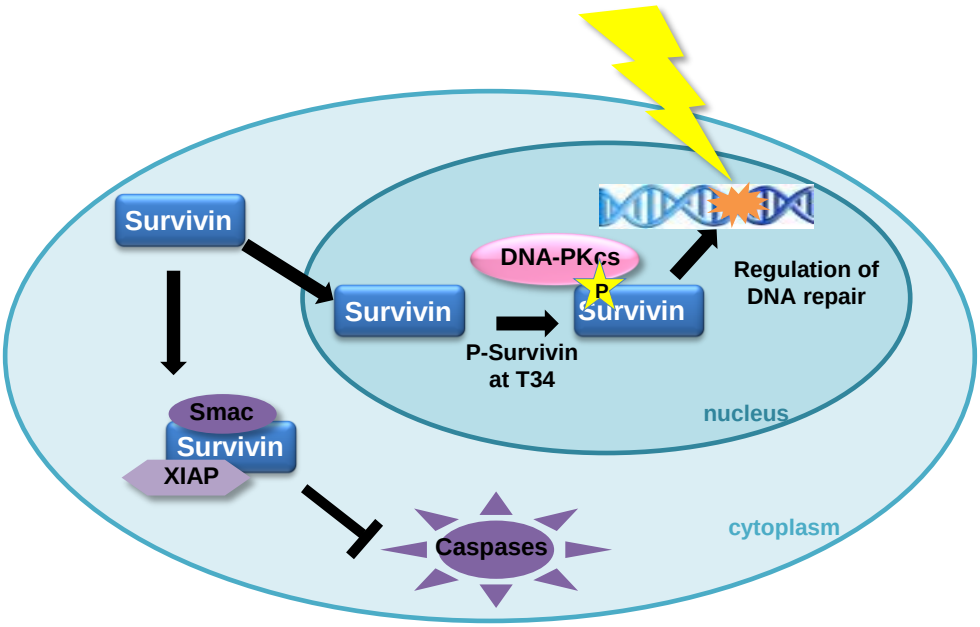


Figure 28: Survivin as a radiation resistance factor. Survivin is here depicted to translocate to the nucleus following irradiation and regulate DNA repair via interaction with DNA repair proteins; an interaction possible only after Survivin phosphorylation at Threonine 34 located within the XIAP binding site of the protein.

It is hereby and for the first time shown that Survivin XIAP binding and T34 phosphorylation sites, but not those of Microtubules and Hsp90, are important for radiation clonogenic survival and regulation of DNA damage repair, at least in part by disrupting interaction with DNA-PKcs. In particular, it is suggested that the DNA repair protein DNA-PKcs interacts with Survivin within the latter's XIAP binding site (amino acids 15-38) and/or only when Survivin is phosphorylated at Threonine 34 (Figure 27).

In addition, XIAP, Microtubules and Hsp90 binding sites as well as the BIR domain were demonstrated to be essential for proper cell cycle regulation, apoptosis inhibition and transmigration capacity of colorectal cancer cells.

To a certain extent, these findings have functionally distinguished some of the roles of Survivin BIR domain, XIAP, Microtubules and Hsp90 binding sites regarding radiation

clonogenic survival, DNA DSBs repair, cell cycle regulation, apoptosis inhibition and transmigration of colorectal cancer cells (Figure 29) (also Table 7 in the appendix).

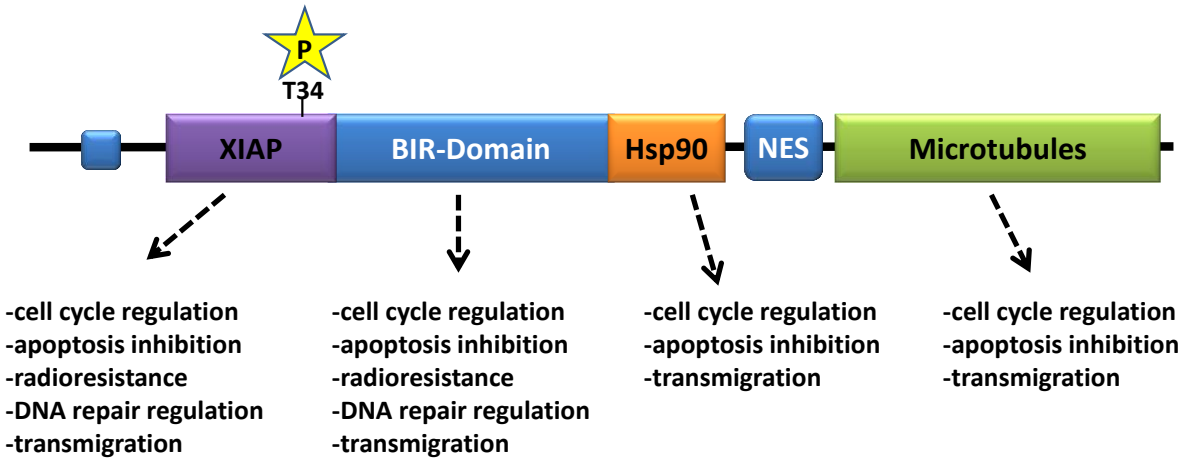


Figure 29: Synopsis of the different binding sites of Survivin and their role in cellular radiation response. Schematic representation of Survivin depicting its BIR domain, XIAP, Hsp90 and Microtubules binding sites each followed by a brief list of their functions.

For future reference, the interaction between DNA repair proteins and mutated constructs of Survivin can be further investigated via DNA-PKcs kinase activity assays and/or mass spectrometry-based quantitative proteomics following stable isotope labeling by amino acids in cell culture (SILAC).

In general, the deletion and phosphorylation mutants of this protein along with the established cell lines stably overexpressing them, represent a tool to further explore the way this protein functions in terms of radiation response and the way it interacts with protein partners such as Smac, Aurora Kinase B, INCENP, XIAP, Hsp90 and members of the DNA repair machinery.

A variety of current preclinical studies employing different strategies targeting Survivin function or expression produce substantial evidence that such an approach can inhibit tumour growth and induce cancer cell-death improving treatment outcome. Hence, there is a number of undergoing clinical trials implementing approaches involving anti-sense

oligonucleotides against Survivin (such as Gataparsen) as well as transcriptional inhibitors (YM155) and immunotherapeutic schemes; the latter using Survivin directed autologous cytotoxic T lymphocytes in recurrent oral and colorectal cancer, myeloma and malignant melanoma [158-161]. According to early clinical data, those antagonists revealed a relatively modest activity having been used as single agents; however, it is believed that if combined with conventional chemotherapeutic drugs, monoclonal antibodies or irradiation, they may well be of an enhanced therapeutic advantage [148].

In summary, the present thesis for the first time showed that overexpression of Survivin T34A or Survivin lacking its XIAP binding site results in a significant radiosensitization and increased levels of unrepaired DNA damage along with no interaction with DNA-PKcs, indicating that these mutations interfere with the ability of colorectal cancer cells to repair DNA damage following irradiation.

To conclude, it is hypothesized that phosphorylation of Survivin on Threonine 34 is essential for cellular radiation survival proving intervention against Survivin function a promising approach to improve the outcome of standard radio-chemotherapeutic treatments.

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Appendix

Table 5: Radiation response variables of 3D grown human SW480 colorectal cancer cells.

Construct Treatment	n	Plating efficiency ± SD [%]	α [Gy^{-1}]	β [Gy^{-2}]	Radiation dose at 50% cell survival [Gy]	SER (vs. co si)
pEGFP						
mock	4	16.98 ± 4.23	0.1470	0.0118	3.65	0.92
co si	7	15.57 ± 5.79	0.1799	0.0078	3.36	1.00
Surv. si	7	8.32 ± 3.45	0.3141	0.0014	2.18	1.54
Surv. wt						
mock	4	14.23 ± 3.91	0.1311	0.0049	4.53	0.95
co si	4	14.58 ± 2.94	0.1531	0.0020	4.29	1.00
Surv. si	4	13.36 ± 2.80	0.1067	0.0099	4.56	0.94
ΔXIAP						
mock	4	10.89 ± 3.24	0.1762	0,0045	3.60	1.03
co si	4	12.24 ± 4.85	0.1866	0.0000	3.72	1.00
Surv. si	4	7.24 ± 2.93	0.2409	0.0045	2.74	1.36
ΔBIR						
mock	4	13.29 ± 5.49	0.1994	0.0000	3.48	1.04
co si	4	12.85 ± 3.84	0.1912	0.0000	3.62	1.00
Surv. si	4	6.59 ± 1.86	0.2836	0.0000	2.44	1.48
ΔMicTub						
mock	4	14.02 ± 3.78	0.1716	0.0000	4.04	0.94
co si	4	15.03 ± 4,47	0.1767	0.0018	3.78	1.00
Surv. si	4	9.61 ± 4.47	0.1130	0.0053	4.97	0.76
ΔHsp90						
mock	4	14.18 ± 3.89	0.1332	0.0063	4.32	1.01
co si	4	13.63 ± 3.75	0.1363	0.0052	4.36	1.00
Surv. si	4	9.33 ± 3.42	0.1158	0.0093	4.42	0.99

The fit of the dose-effect curves was calculated by means of the linear-quadratic model ($\ln SF = -\alpha \times D - \beta \times D^2$). The radiation dose at 50% survival was calculated by transforming the linear quadratic equation ($SF = \exp[-\alpha \times D - \beta \times D^2]$) with α and β values of the individual survival curves. The sensitizer enhancement ratio at 50% survival (SER) was calculated by dividing the radiation dose at 50% survival of control siRNA (co si) treated cells by the

radiation dose at 50% survival of mock or Survivin siRNA (Surv. si) treated cells. D, radiation dose [Gy]; n, number of independent experiments; SD, standard deviation; SF, surviving fraction; pEGFP, enhanced green fluorescent protein expressing control; Surv. wt, Survivin wild type; ΔXIAP, Survivin X-linked inhibitor of apoptosis protein binding site; ΔBIR, baculovirus IAP repeat domain; ΔMiCTub, Microtubules binding site; ΔHsp90, Hsp90 binding site deletion mutants stably expressed in SW480 cells.

Table 6: Radiation response variables of alternative clones and phospho-mutants of 3D grown human SW480 colorectal cancer cells.

Construct Treatment	n	Plating efficiency ± SD [%]	α [Gy^{-1}]	β [Gy^{-2}]	Radiation dose at 50% cell survival [Gy]	SER (vs. co si)
pEGFP #2						
co si	4	11.18 ± 4.15	0.1574	0.0123	3.46	1.00
Surv. si	4	4.14 ± 1.31	0.3477	0.0019	1.97	1.76
Surv. wt #2						
co si	4	10.43 ± 3.10	0.1482	0.0124	3.59	1.00
Surv. si	4	9.68 ± 3.76	0.1748	0.0121	3.24	1.11
ΔXIAP #2						
co si	4	8.29 ± 1.57	0.2188	0.0008	3.13	1.00
Surv. si	4	3.62 ± 1.96	0.2687	0.0268	2.13	1.47
S20A						
co si	3	8.48 ± 3.31	0.1407	0.0371	2.82	1,00
Surv. si	3	3.92 ± 1.63	0.3036	0.0000	2.28	1,24
S20D						
co si	3	14.90 ± 3.39	0.0733	0.0220	4.19	1.00
Surv. si	3	7.24 ± 2.39	0.1151	0.0219	3.58	1.17
T34A						
co si	6	11.15 ± 3.47	0.3076	0.0064	2.16	1.00
Surv. si	6	8.00 ± 1.59	0.3202	0.0194	1.94	1.11
T34D						
co si	6	13.74 ± 5.47	0.1510	0.0155	3.40	1.00
Surv. si	6	10.42 ± 3.84	0.2417	0.0054	2.70	1.26
T34A #2						
co si	3	5.37 ± 1.20	0.4427	0.0000	1.57	1.00
Surv. si	3	6.86 ± 2.05	0.3396	0.0263	1.79	0.87
T34D #2						
co si	3	11.40 ± 1.07	0.1885	0.0156	2.95	1.00
Surv. si	3	8.97 ± 0.88	0.3153	0.0073	2.10	1.41
T117A						
co si	3	0.15 ± 0.02	0.1834	0.0137	3.07	1.00
Surv. si	3	0.11 ± 0.03	0.1296	0.0202	3.47	0.89
T117D						
co si	3	0.12 ± 0.03	0.2305	0.0087	2.73	1.00
Surv. si	3	0.05 ± 0.02	0.2404	0.0098	2.61	1.05

The fit of the dose-effect curves was calculated by means of the linear-quadratic model (ln SF = $-\alpha \times D - \beta \times D^2$). The radiation dose at 50% survival was calculated by transforming the linear quadratic equation (SF = exp [$-\alpha \times D - \beta \times D^2$]) with α and β values of the individual survival curves. The sensitizer enhancement ratio at 50% survival (SER) was calculated by dividing the radiation dose at 50% survival of control siRNA (co si) treated cells by the radiation dose at 50% survival of mock or Survivin siRNA (Surv. si) treated cells. D, radiation dose [Gy]; n, number of independent experiments; SD, standard deviation; SF, surviving fraction; pEGFP, enhanced green fluorescent protein expressing control; Surv. wt, Survivin wild type; ΔXIAP, Survivin X-linked inhibitor of apoptosis protein binding site; S20A, S20D, T34A, T34D, T117A, T117D mutants stably expressed in SW480 cells.

Table 7: Synopsis of observed effects of Survivin mutants on cellular radiation response

Survivin mutation	Effects on cellular radiation response or transmigration
Survivin ΔXIAP	G2/M arrest, increased values of caspase 3/7 activity, increased radiosensitivity, increased levels of DNA DSBs, no precipitation with DNA-PKcs, decreased levels of transmigration
Survivin ΔBIR	G2/M arrest, increased values of caspase 3/7 activity, increased radiosensitivity, increased levels of DNA DSBs, decreased levels of transmigration
Survivin ΔMicTub	G2/M arrest, increased values of caspase 3/7 activity, decreased levels of transmigration
Survivin ΔHsp90	G2/M arrest, increased values of caspase 3/7 activity, decreased levels of transmigration
Survivin S20A	radiosensitivity
Survivin S20D	-
Survivin T34A	increased radiosensitivity, increased levels of DNA DSBs, no precipitation with DNA-PKcs
Survivin T34D	-
Survivin T117A	-
Survivin T117D	-

Abbreviations

°C	degree Celsius
γH2AX	gamma H2A histone, member X
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
2D	two-dimensional
3D	three-dimensional
53BP1	p53-binding protein 1
A	Alanine
ACF	Aberrant crypt focus
AIP	Aryl hydrocarbon receptor-interacting protein
APC	Adenomatous polyposis coli gene
ASO	Anti-sense oligonucleotide
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia rad3-related protein
BSA	Bovine serum albumin
BIR	Baculovirus IAP repeat
BRCA1	Breast cancer 1
BRAF	proto-oncogene B-Raf
CARD	Caspase-associated recruitment domain
CDK1	Cyclin-dependent kinase 1
CIN	Chromosomal instability
CIMP	CpG island methylator phenotype
cIAPs	Cellular inhibitor of apoptosis proteins
CRC	Colorectal cancer
CO_2	Carbon dioxide

CFA	Colony forming assay
CPC	Chromosomal passenger complex
c-Ha-Ras	V-Ha-ras Harvey rat sarcoma viral oncogene homologue
c-Myc	V-myc myelocytomatosis viral oncogene homologue (avian)
D	Aspartic acid
DAPI	4',6-diamidino-2-phenylindole
DIABLO	Direct IAP-binding protein with low pI
DIAP1	Drosophila IAP 1
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DNA DSBs	DNA Double-strand breaks
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FOXO1	Forkhead box O1
G2	Growth 2 phase (of the cell cycle)
GTP-binding protein	guanosine nucleotide-binding protein
HBXIP	Hepatitis B virus X-interacting protein
HIF-1α	Hypoxia inducing factor 1 alpha
HR	Homologous recombination
Hsp90	Heat shock protein 90
IAP	Inhibitor of apoptosis protein
IBM	IAP-binding motif
IgG	Immunoglobulin G

IP	Immunoprecipitation
IF	Immunofluorescence
INCENP	Inner centromere protein
KRAS	V-Ki-ras2 Kirsten rat sarcoma (viral oncogene homolog)
LB medium	Luria Bertani medium
Lys	Lysine
M	Mitotic phase of the cell cycle
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MDC1	Mediator of DNA damage checkpoint protein 1
mg	milligram
min	minute
MicTub	Microtubules
MMR	Mismatch repair
MRN	Mre11, Rad50 and Nbs1 complex
mRNA	Messenger RNA
MSI	Microsatellite instability
mTOR	Mammalian target of rapamycin
n	Number of experiments
NES	Nuclear export signal
ng	nanogram
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end joining
NSCLC	Non-small cell lung cancer
p53	Tumour suppressor protein 53
PCR	Polymerase Chain Reaction
PKA	Protein kinase A

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Plk1	Polo-like kinase 1
PTEN	Phosphatase and tensin homolog deleted from chromosome ten
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
RING	Really Interesting Gene
S	serine
SDS	Sodium dodecylsulfate
siRNA	Small interfering RNA
SIRT1	Silent mating type information regulation 2 homolog 1
Smac	second mitochondria-derived activator of caspases
SMAD2 (or SMAD4)	Mothers against decapentaplegic homolog 2 (or 4)
Src	proto-oncogene c-Src
STAAT3	Signal transduction and activator of transcription 3
UBA	Binding site for polyubiquitinated proteins
TBS buffer	Tris-buffered saline
TAE buffer	Tris-Acetate-EDTA buffer
TCF4	Transcription factor 4
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
TRIS	Tris hydroxymethyl aminomethane
TP53	Tumour protein 53 (gene)
wt	Wild-type

Wnt	wingless-related integration site
XAF1	XIAP-associating factor 1
XIAP	X-chromosome linked inhibitor of apoptosis protein
XRCC4	X-ray repair cross-complementing protein 4

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