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Dynamische Lokalisation des tumorrelevanten Proteins Survivin

Molekulare Mechanismen, therapeutisches und prognostisches Potential

Kumulative Habilitationsschrift
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Dynamic Localisation of the tumor-relevant Protein Survivin
Molecular mechanisms, therapeutic and prognostic potential

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Verzeichnis der in dieser kumulativen Habilitationsschrift zusammengefassten Arbeiten

Index of publications summarized in this professorial dissertation

I. Stauber R.H., Rabenhorst U., Rekik A., Engels K., Bier C., & **Knauer S.K.** (2006). Nucleocytoplasmic Shuttling and the Biological Activity of Mouse Survivin Is Regulated By an Active Nuclear Export Signal. Traffic, 7, p. 1461-72.

II. **Knauer S.K.**, Bier C., Habtemichael N., & Stauber R.H. (2006). The Survivin-Crm1 interaction mediates targeting of the chromosomal passenger complex to the centromere. EMBO reports, 12, p.59-65.

III. Engels K.*, **Knauer S.K.***, Metzler D., Simf C., Struschka O., Bier C., Mann W., Kovács A.F., & Stauber R.H. (2006). Dynamic Intracellular Survivin in Oral Squamous Cell Carcinoma – Underlying Molecular Mechanism and Potential as an Early Prognostic Marker. Journal of Pathology, 211(5), p. 532-40.*, authors contributed equally to this work.

IV. **Knauer S.K.**, Krämer O.H., Knösel T., Engels K., Rödel F., Kovács A.F., Dietmaier W., Klein-Hitpass L., Habtemichael N., Schweitzer A., Brieger J., Rödel C., Mann W., Petersen I, Heinzel T., & Stauber R.H. (2007). Nuclear export is essential for the tumor promoting activity of survivin. FASEB J, 1, p.207-16.

V. **Knauer S.K.**, Mann W., & Stauber R.H. (2007). Survivin's Dual Role - An Export's View. Cell Cycle., 6(5), p. 518-21 (Review article).

VI. **Knauer S.K.**, Bier C., Schlag P., Fritzmann J., Dietmaier W., Rödel F., Klein-Hitpass L., Kovács A.F., Döring C., Hansmann M.-L., Hofmann W.-K., Kunkel M., Brochhausen C., Engels K., Lippert B., Mann W., & Stauber R.H. (2007). The survivin splice variant survivin-3B is cytoprotective and can function as a chromosomal passenger complex protein. Cell Cycle, 12(6), 6(12), p. 1502-9.

VII. Lippert B.M.*, **Knauer S.K.**,* Fetz V., Mann W., & Stauber R.H. (2007). Dynamic survivin in head and neck cancer: Molecular mechanism and therapeutic potential. Int J Cancer, 121(6), p. 1169-74. (Review article) .*, authors contributed equally to this work.

VIII. Stauber R.H., Mann W., & **Knauer S.K.** (2007). Nuclear and Cytoplasmic Survivin: Molecular Mechanism, Prognostic, and Therapeutic Potential. Cancer Research, 67(13):5999-6002. (Review article).

1 ZUSAMMENFASSUNG

Karzinome des Darm- und Kopf-Hals-Bereichs gehören mit zu den häufigsten Krebserkrankungen. Trotz guter Therapieerfolge durch Früherkennung und innovative Behandlungsmethoden entwickeln viele Patienten nach der Erstbehandlung ein Rezidiv, und oftmals treten Metastasen auf. Für Unterschiede im Therapieansprechen werden hauptsächlich zelluläre Resistenzmechanismen verantwortlich gemacht. Von wissenschaftlicher und klinisch herausragender Bedeutung ist es daher, molekulare Mechanismen und Proteine zu entschlüsseln, welche kausal an der Krebsentstehung und Therapieresistenz beteiligt sind. Anschließend kann nach genetischen oder pharmakologischen Inhibitoren gesucht werden, welche spezifische Eigenschaften dieser Faktoren blockieren und somit als Leitstrukturen für die Entwicklung neuer Krebsmedikamente (*„from bench to bedside“*) dienen können.

Unsere Körperzellen sind in unterschiedliche Bereiche unterteilt, wodurch einerseits die verschiedenen in der Zelle ablaufenden Prozesse besser reguliert werden können, andererseits aber auch ein ausgeklügeltes Transportsystem für den Stoffaustausch erforderlich wird. Die stattfindenden Transportvorgänge können von der Zelle auf mannigfaltige Weise reguliert werden, wobei eine Fehlsteuerung dieser Prozesse jedoch auch zur Entstehung von Krankheiten wie beispielsweise Krebs beitragen kann. Ein Eiweiß, welchem eine entscheidende Rolle bei der Krebsentstehung zugeschrieben wird, ist das Protein Survivin, ein Mitglied der "Inhibitoren der Apoptose Protein"-Familie und zugleich ein Regulator der Zellteilung.

Durch den Einsatz innovativer Techniken der Mikroskopie und der Computer-gestützten Mikroinjektion in lebende Zellen konnten wir zeigen, dass Survivin aktiv aus dem Zellkern transportiert wird (*I,* Stauber *et al.* 2006; *II,* Knauer *et al.* 2006). Der Kernexport dieses in beinahe allen Krebsarten vorkommenden Eiweißes war nicht nur für den Schutz von Krebszellen gegenüber Radio- und Chemotherapie, sondern auch für den korrekten Ablauf der Zellteilung verantwortlich (*II,* Knauer *et al.* 2006; *IV,* Knauer *et al.* 2007b). Survivin wird also durch sein Transportsignal auch an die Zellteilungsmaschinerie geleitet, wodurch es die Zelle sicher durch die Teilung bringt - es übt somit eine duale Funktion aus (*V*, Knauer *et al.* 2007c; *VIII*, Stauber *et al.* 2007).

Die Hypothese, dass Survivin im Zellkern unfunktionell zu sein scheint, wird auch durch die Analyse von Tumorproben von Kopf-/Hals- und Kolonkarzinom-Patienten gestützt. Es zeigte sich, dass Patienten mit hauptsächlich nukleärem Survivin im Tumorgewebe deutlich bessere Überlebenschancen hatten als Patienten mit viel Survivin im Zytoplasma (*III,* Engels *et al.* 2007; *VII,* Lippert *et al.* 2007).

Die funktionelle Bedeutung des nukleären Exports von Survivin wird durch weitere Arbeiten untermauert, welche die Anwesenheit eines Exportsignals als wichtige Voraussetzung für die Funktion der Survivin-Isoformen aufzeigen. Obwohl die genaue biologischen Funktion der insgesamt fünf postulierten Spleißformen sowie deren Beitrag zur Krebsentstehung noch immer kontrovers diskutiert werden, konnte in umfassenden Studien in verschiedenen Tumorentitäten gezeigt werden, dass das Wildtyp (WT) Survivin-Protein die vornehmlich nachweisbare und tumorfördernde Form darstellt. Außerdem scheinen die weiteren Survivin-Varianten nicht in der Lage zu sein, die Aktivität des WT Proteins signifikant *in trans* zu modulieren (*VI,* Knauer *et al.*

2007a). Diagnostische Bemühungen wie auch pharmakogentische Interventionstrategien sollten sich demnach hauptsächlich auf das Survivin WT-Protein konzentrieren.

Die differentielle Überexpression von Survivin in den meisten Krebserkrankungen sowie dessen duale tumorfördernde Funktion weisen Survivin als ideale Zielstruktur für selektive Therapiestrategien aus. Die in dieser Habilitationsarbeit vorgestellten Ergebnisse erlauben, eine spezifische Inhibition des Kernexports von Survivin als neuartiges therapeutisches Prinzip zu postulieren. Könnte man das Protein in den entsprechenden Tumoren durch therapeutisch wirksame Substanzen vom Zytoplasma in den Zellkern treiben, sollten die Tumorzellen gegenüber derzeitigen Krebstherapien sensitiviert werden und sich so die Überlebensrate der Krebspatienten erhöhen. Da Survivin aufgrund seiner oben beschriebenen dualen Rolle zugleich zwei verschiedene Angriffspunkte bietet, könnte durch die gerichtete Interferenz mit dem Kernexport von Survivin nicht nur die Resistenzbildung sondern auch die Wachstumsrate von Krebszellen gehemmt werden - im Sinne eines "*Exportstopp für Krebszellen*" als neuartiges therapeutisches Prinzip (*V,* Knauer *et al.* 2007c; *VIII,* Stauber *et al.* 2007).

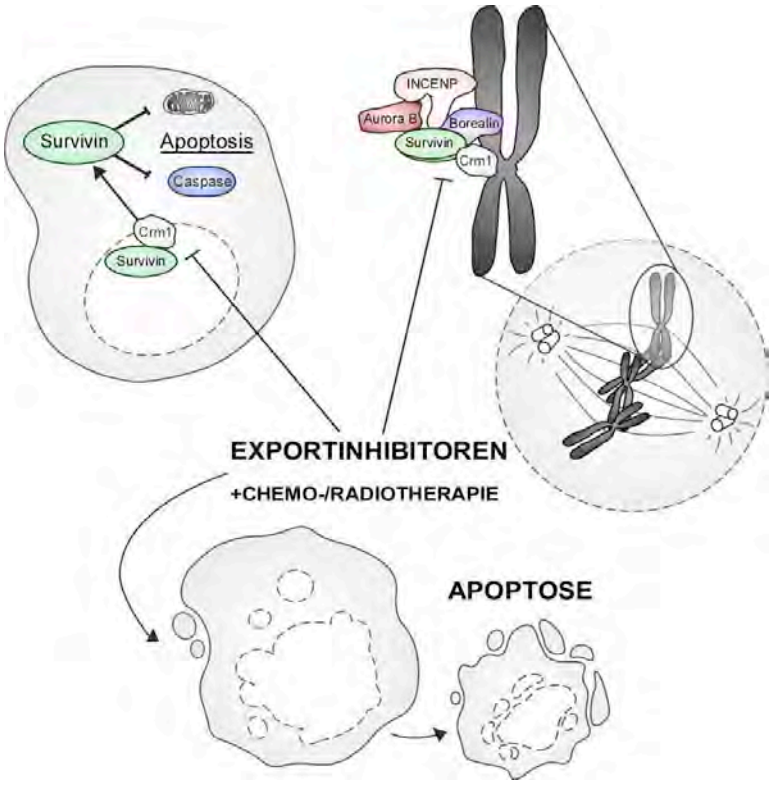

Abbildung 1. **Survivin's dynamische Lokalisation und Wechselwirkung mit dem Exportrezeptor Crm1 sind essentiell für die duale Aktivität von Survivin.** In Interphase-Zellen erlaubt die Export-vermittelte zytoplasmatische Lokalisation von Survivin eine effiziente Inhibition der Apoptosemaschinerie. Während der Mitose rekrutiert die Survivin-Crm1 Interaktion den CPC zu den Zentromeren. Die Verhinderung der Survivin-Crm1 Wechselwirkung durch niedermolekulare Exportinhibitoren, könnte in Kombination mit Radio-/Chemotherapie zu einer erhöhten Apoptose der Tumorzellen führen.

1 SUMMARY

Head&neck and colon cancer are amongst the most common malignancies in humans worldwide. Over the last decades, diagnosis and disease management have improved significantly, but not long-term survival rates. Loco-regional recurrence after therapy and metastasis are the major cause of death. Consequently, identification of molecular markers and biological mechanism that signal increased risk of treatment failure or that can be exploited by targeted therapy is actively pursued by academia and industry.

The cells of our body are divided into different compartments what on the one hand allows a highly efficient regulation of cellular processes, but on the other hand require sophisticated molecular transport systems. Regulated nucleo-cytoplasmic localization is exploited by the cell as an attractive way to control the activity and stability of regulatory proteins. However, malfunction of these events may contribute to the nascency of diseases like cancer. One protein attributed a crucial role in tumorigenesis is "survivin", a member of the "inhibitors of apoptosis protein" family, which at the same time, functions as a modulator of mitosis.

Corroborating experimental approaches including innovative microscopic techniques and computer-guided microinjection in living cells provided evidence that survivin is actively exported from the nucleus to the cytoplasm (*I,* Stauber *et al.* 2006; *II,* Knauer *et al.* 2006). Nuclear export of survivin, which we found overexpressed in several tumor entities, was not only essential to protect cancer cells from radio- and chemotherapy-induced apoptosis, but also for survivin's function during mitosis (*IV,* Knauer *et al.* 2007b). The exported-mediated high cytoplasmic concentration of survivin promotes its cytoprotective function by facilitating its interplay with the apoptotic machinery in interphase cancer cells. During mitosis, survivin is tethered to the mitotic machinery also by its innate transport signal, thereby escorting the cell securely through division (*V,* Knauer *et al.* 2007c; *VIII,* Stauber *et al.* 2007). Our hypothesis that preferential cytoplasmic survivin represents "cytoprotective survivin", whereas nuclear survivin signals "impaired survivin function" is further supported by patient data. In head&neck and colon cancer patients, preferential nuclear survivin in tumor cells correlated with favorable disease, whereas high cytoplasmic survivin was associated with poor survival (*III,* Engels *et al.* 2007; *VII,* Lippert *et al.* 2007).

The significance of nuclear export for survivin's functions is further underlined by our findings that the presence of a nuclear export signal in survivin splice variants is critical for their biological activity. Although the exact biological functions of the five proposed survivin splice variants and their contribution to cancer progression are still controversially discussed, we could show that wildtype (wt) survivin was the predominant and mostly tumor promoting form detectable in different tumor entities. Also, the survivin isoforms are not versed to significantly modulate the activity of the wt protein *in trans* (*VI,* Knauer *et al.* 2007a). Consequently, diagnostic efforts as well as pharmacogenetic intervention strategies should focus on wt survivin.

Since the survivin 'network' is exploited in virtually every human cancer, survivin is currently regarded as a promising target for rational therapy. Summarizing the data from this thesis, it seems plausible that molecular decoys selectively targeting the nuclear export of survivin might be of therapeutic relevance. Since survivin's dual functions provide two different points of attack at one time, targeted interference with its nuclear export should not only prevent therapy resistance but also cancer growth - introducing a "*NO GO! - for Cancer Cells*" as a novel therapeutic principle (*V,* Knauer *et al.* 2007c; *VIII,* Stauber *et al.* 2007).

2 INTRODUCTION

The survival of a multicellular organism depends on the intactness of size and function of the different organs. Homeostasis is orchestrated by information kept in genes. Absence of proper genetic information or its deregulation may lead to various diseases.

More than hundred years ago, *Charles Darwin* presented his theory of evolution, explaining how different species evolve due to the fact that "the fittest survive". Also in the body of a multicellular organism, "evolution" can take place. A cell that manages to get a growth advantage due to deregulation of genes, will be fitter than its surrounding cells, and might evolve into a tumor. Depending on the cell type, the environment, and the types of deregulation of cellular information, the reasons why cells evolve into cancer cells are different. Thus, a cancer disease is recognized as a growing tumor, but the underlying reasons for its occurrence and the optimal treatment for tumor elimination might be different. However, in all tumors, the information regulating growth and death are deregulated. Therefore, there is a general requirement for increased knowledge about mechanisms regulating life and death in different tumor cells, and knowledge on how these mechanisms can be manipulated by various types of medicines is essential.

2.1 Cancer

It was the ancient Greek physician *Hippocrates* who denominated tumors "*carcinos*", Greek for crab or crayfish, as the appearance of a solid malignant tumor vaguely resembles the shape of a crab. He later added the suffix *"-oma"*, Greek for swelling, giving the name "*carcinoma*". Half a century later, *Aulus Cornelius Celsus*, a Roman encyclopedist, translated the Greek term into the Latin word *"cancer"*, also meaning crab. The Greek physician *Galen* used *"oncos"*, the Greek term for swelling, which was previously used specifically for benign tumors, to describe all types of cancer, laying the foundation for the modern word *"oncology"*.

In 2006, an estimated 1.7 million people died from cancer in Europe, and 3.2 million cases were diagnosed (Ferlay *et al.* 2007). This makes cancer the second most common death cause following cardiovascular diseases. Over the next decades, the number of diagnoses is expected to increase further, as longevity climbs in developing nations. The chemotherapeutic agents currently used for therapy are the drugs with the least effective therapeutic index of all medicine. Thus, an effective dose of a wide variety of anticancer agents is restricted by their non-selective, highly toxic effect. Especially concerning longevity increase, this states an acute problem because chemotherapy-related toxicity is far more common in older patients. Therefore there is an urgent need to develop more specific and less toxic cancer therapies.

To achieve this, however, a more detailed understanding of the molecular mechanisms leading to malignant transformation and therapy resistance is of utmost importance. Cancer is a very complex disease generated by multiple genetic alterations, and can be viewed as the loss of cooperative cell behaviors normally facilitating multicellularity, including the formation of tissues and organs. (for review see Hanahan & Weinberg 2000; Vogelstein & Kinzler 2004). Malignant, invasive tumors are

characterized by phenotypic changes at the cellular level as the essential hallmarks of cancer: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, block of cellular differentiation, evasion of apoptosis, genetic instability, limitless replicative potential, sustained angiogenesis, tissue invasion and metastatic potential (see Fig. 2.1).

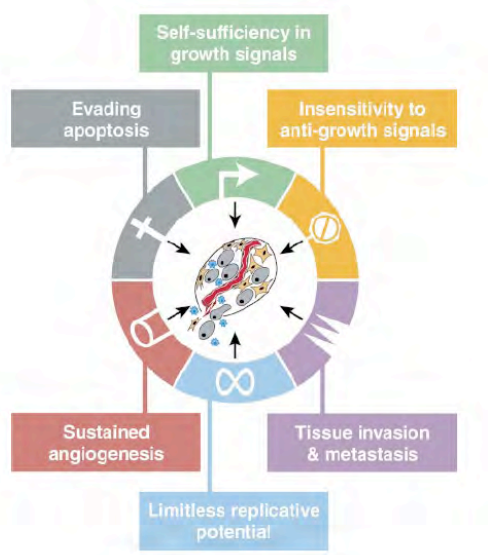

Figure 2.1. **Acquired properties of cancer cells.** See text for details.
(Figure from Hanahan & Weinberg 2000)

Only with holistic clarity of mechanism, cancer prognosis and treatment will become a rational science. Anticancer drugs should be targeted to each of the hallmark capabilities of cancer, used in appropriate combinations and in concert with sophisticated technologies.

2.2 Apoptosis

Apoptosis, also called programmed cell death, is a mechanism that allows cells to self-destruct when stimulated by an appropriate trigger. This program is activated for various reasons, e.g. when the cell is no longer needed within the body or when it becomes a threat to the health of the organism. Thus, apoptosis is a key component in the development and maintenance of tissues within multicellular organisms, providing a tightly regulated and selective mechanism for the deletion of superfluous, infected, mutated or aged cells. Basically, apoptosis is a normal physiological process that offsets cell proliferation (for reviews see Igney & Krammer 2002; Jesenberger & Jentsch 2002). Proteins involved in this process are evolutionarily conserved. The apoptotic executive machinery can be activated via extrinsic and intrinsic pathways, with its center constituted by a family of cysteinyl proteases, termed caspases (cysteinyl aspartate-specific proteases). Further regulation is achieved by involvement of the Bcl-2 as well as the "inhibitors of apoptosis" protein (IAP) family (see Fig. 2.2), directly or indirectly interfering with the apoptotic pathway.

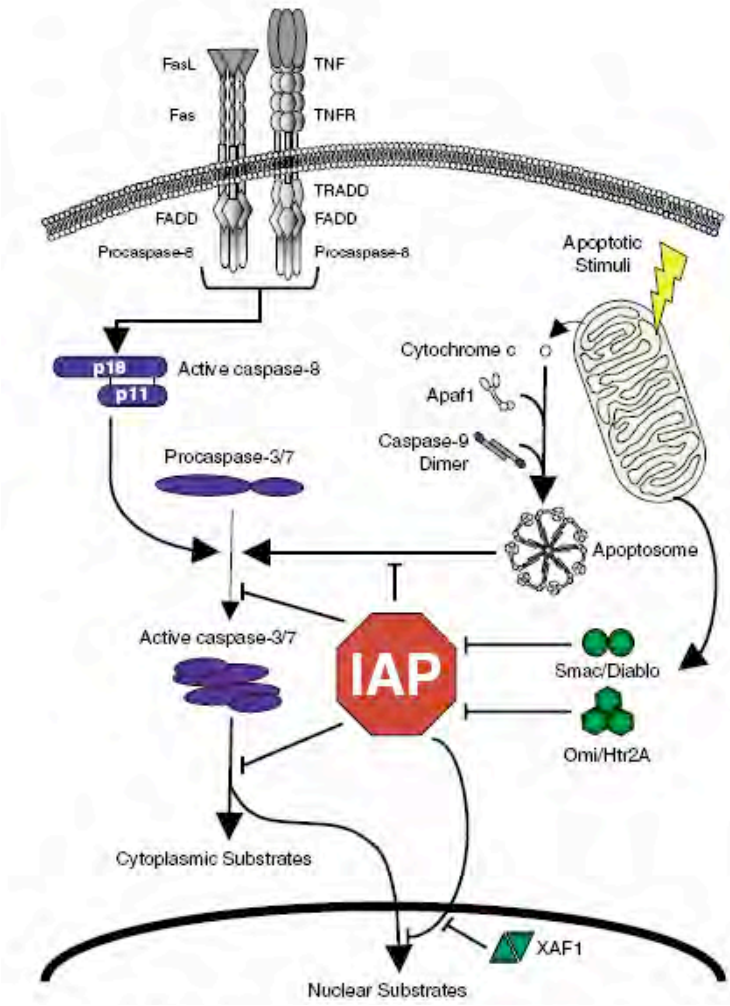

Figure 2.2. **Role of the IAPs in regulating apoptotic pathways.** Inhibition of both initiator and effector capases uniquely situates the IAPs at the junction of both major pathways (Figure from (Liston *et al.* 2003).

Dysregulation of apoptosis contributes to a variety of pathologic conditions, including cancer (reviewed in Meier *et al.* 2000; Gerl & Vaux 2005; Knauer 2005). As tumor cells show a disturbed equilibrium between proliferation and apoptosis, conventional cancer therapies take advantage of this apoptotic mechanism by employing ionizing radiation or chemotherapeutic drugs to damage DNA and induce selective apoptosis of rapidly growing cells. Defective apoptosis in tumor cells contributes to the survival of cells beyond intended lifespan, allowing for accumulation of genetic alterations that deregulate cell proliferation, interfere with differentiation, promote angiogenesis and increase cell motility and invasiveness during tumor progression. Defects of apoptosis regulators are indeed often observed in human cancers, providing tumor cells with a survival advantage and rendering them resistant to chemotherapeutic drugs. Thus, many therapeutic strategies directly targeting apoptosis are being pursued preclinically or clinically (Reed & Wilson 2003).

2.3 The "inhibitors of apoptosis" (IAP) protein family

Since the discovery of the first IAP in baculoviruses (Clem *et al.* 1991; Clem & Miller 1994), a multitude of related proteins have been described in virtually all eukaryotes. In addition to their presence in these organisms, their scope of function and activity has also diversified with evolution. The IAP family can suppress apoptosis by interacting with, and inhibiting the enzymatic activity of caspases (Deveraux & Reed 1999). IAPs have also been implicated in cell division, cell cycle progression and signal transduction (for review see Schimmer *et al.* 2004).

The investigation of their importance in cell survival signaling has intensified greatly in recent years with the observation that in humans, IAP malfunction contributes to various diseases. The identifying characteristic of the IAP family members lies not singularly in their ability to prevent apoptosis, but rather in the presence of a common structural motif. This novel domain, termed the baculovirus IAP repeat (BIR), domain is a cysteine- and histidine-rich sequence motif of 70 aa length which has been identified as a novel zinc-binding fold and has been shown to bind to and inhibit caspases (Deveraux *et al.* 1997; Roy *et al.* 1997; Deveraux *et al.* 1998; Takahashi *et al.* 1998; Deveraux & Reed 1999). In addition to the BIR domain, a number of IAPs contain a carboxyl-terminal RING zinc finger domain. Some IAPs also possess a caspase recruitment domain (CARD), also present in many of the adapter molecules controlling apoptosis signaling. Membership of the IAP family requires the presence of at least one of these domains.

So far, eight human IAPs have been identified (see Fig. 2.3), including NIAP, XIAP, cIAP1, cIAP2, livin and survivin (Deveraux *et al.* 1998; Chen *et al.* 1999; Schimmer *et al.* 2004). At least cIAP1, cIAP2 and XIAP have been shown to directly inhibit caspase activity. XIAP inhibits caspase-8-induced protease activity at the level of caspase-3 and caspase-7 whereas cytochrome c induced activation is additionally prevented upstream of the effector caspases by direct inhibition of caspase-9 processing.

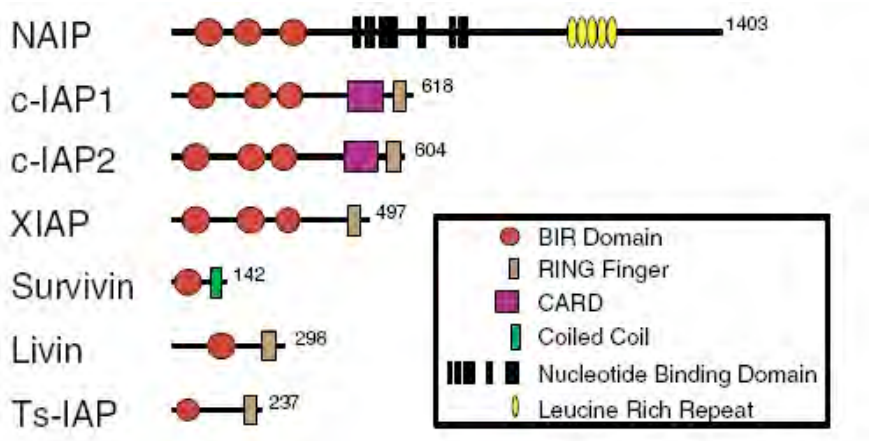

Figure 2.3. **Domain structure of the IAP family.** Individual domains are drawn to scale. Abbreviations are as follows: BIR: baculoviral IAP repeat; CARD: caspase recruitment domain; RING: RING zinc finger; NOD: nucleotide-binding oligomerization domain; and LRR: leucine-rich repeats (Figure modified from Liston *et al.* 2003).

2.4 Survivin

Survivin, the smallest mammalian member of the IAP family (Salvesen & Duckett 2002), contains a single BIR domain and exists as a stable homodimer in solution (see Fig. 2.4, Chantalat *et al.* 2000; Muchmore *et al.* 2000; Verdecia *et al.* 2000; Sun *et al.* 2005). A single-copy survivin gives rise to the four alternatively spliced survivin transcripts survivin-2B, -3B, ΔEx-3 and -2α (Altieri 2003c; Caldas *et al.* 2005a; Caldas *et al.* 2005b; Fangusaro *et al.* 2005), and references within). Although, the low molecular weight would allow survivin to access intracellular compartments by passive diffusion, regulated subcellular localization has also been suggested for survivin (reviewed in Altieri 2004). The proposed existence of transport signals in survivin and its isoforms is shown in Figure 2.4.

Figure 2.4. **Clustal-based alignment of survivin and its splice variants.** Amino acid positions and exons are indicated. NES: Nuclear export signal. Basic cluster I/II indicate potential NLSs. (Figure from Knauer *et al.*, 2007).

Survivin is cell cycle regulated, and involved in both control of apoptosis and regulation of cell division (Li *et al.* 1998). Survivin is preferentially expressed in fetal tissues, suggesting that it plays a pivotal role in development (Ambrosini *et al.* 1997). It is undetectable in most normal adult tissues, but is highly expressed in cancer. As its expression correlates with reduced tumor cell apoptosis, abbreviated patient survival (Adida *et al.* 1998; Kawasaki *et al.* 1998; Moore 2001; Smith *et al.* 2001), accelerated rates of recurrences, and increased resistance to chemo- and radiotherapy, major therapeutic and prognostic interest has been focused on survivin (see Altieri 2003b; Altieri 2003a; Li 2003, and references within). The protective effect of survivin is supposed to be due to its caspase-binding capacity and to depend also on its spindle association during cell cycle progression (Li *et al.* 1998; Li *et al.* 1999). However, critical gaps in the molecular understanding of the survivin pathway still exist that have hampered its full exploitation for cancer therapeutics.

Besides its role as an IAP, survivin, as a member of the chromosomal passenger complex (CPC) (Lens *et al.* 2006), acts as a mitotic regulator (Li & Altieri 1999; Uren *et al.* 1999).

2.5 Mitosis

To ensure their survival and to create multicellular organisms, cells multiply by dividing. Cell division is a very complex process that involves every single substructure of the cell. As the genetic material has to be fully segregated between the mother and the daughter cell before cytokinesis takes place, all cellular events related to division have to be precisely monitored and tightly coordinated in space and time (see also Knauer 2005).

Defined as the set of events that leads to the duplication of a cell, the cell cycle is one of the most comprehensively studied biological processes, particularly given its importance for growth and development and its implication in many human disorders (for review see Harper & Brooks 2005). Early light microscopic studies recognized that cell division was preceded by mitosis, during which cells condensed their chromosomes, aligned them on a microtubular spindle and segregated sister chromatids to opposite poles of the cell. Interphase, the interval between succeeding mitoses, remained a mystery until DNA was discovered to be the source of information stored in chromosomes. Chromosome duplication was then detected and shown to occur during a narrow window of time during interphase (Howard & Pelc 1951),which split interphase into three intervals: G1, the gap between mitosis and the onset of DNA replication; S phase, the period of DNA synthesis, where the chromosomes are faithfully duplicated; and G2, the gap between S and M phase. Preparations for S and M phase take place in this preceding gap phases. Under unfavorable environments, cells can exit the G1 phase and enter the quiescent G0 phase, from where they can return to the cycle through the G1 phase when environmental cues permit. Mitosis, the process by which a complete copy of the duplicated genome is precisely segregated by the microtubule spindle apparatus into two daughter cells, is an extraordinarily complex biological process. Given that the survival of a cell depends on the accuracy of mitosis, an elaborate control system using multiple fidelity-monitoring checkpoints has evolved to ensure correct temporal and spatial coordination of this process. Missegregation of chromosomes results in aneuploidy, something that is frequently found in cancers, suggesting that the machinery surveying the chromosome segregation process has somehow been compromised during the development of these tumors. One of the cell cycle checkpoints, the mitotic spindle checkpoint, has also been shown to be defective in cancers with chromosomal instability.

Mitosis comprises different steps - prophase, prometaphase, metaphase, anaphase and telophase (see Figure 2.6) - and usually ends with cell division (cytokinesis). At prophase, chromosome condensation begins, centrosomes separate and the nuclear envelope breaks down. During prometaphase, chromosomes are captured by microtubules growing from the separated centrosomes and bi-orient, congressing to the center of the spindle at metaphase. Chromosome alignment is finished with the end of metaphase. Anaphase marks the loss of cohesion between sister chromatids and their movement to opposite spindle poles, which move apart to further separate daughter nuclei reforming in telophase prior to cytokinesis, and the return to interphase.

2.6 Chromosomal passenger proteins

The Chromosomal Passenger Complex corrects attachment errors between chromosomes and the mitotic spindle, regulates the quality-control checkpoint, and ensures the correct completion of cytokinesis (Lens *et al.* 2006). It is highly conserved among species and consists of at least four members: AuroraB, INCENP, Survivin and Borealin. In many studies it hast been shown that these are involved in early mitosis processes like monitoring the generation of tension between two sister chromatids. Furthermore, in higher eukaryotes they positively regulate cytokinesis. This protein complex shows a complex localization pattern during the canonical morphological phases of mitosis (see Fig. 2.6). In prophase, proteins of this complex accumulate in the nucleus to associate along the length of condensing chromosomes. Subsequently, they accumulate at the inner centromere of prometa- and metaphase chromosomes spindle midzone of early anaphase cells, and the midzone and equatorial cortex of late anaphase, telophase, and dividing cells.

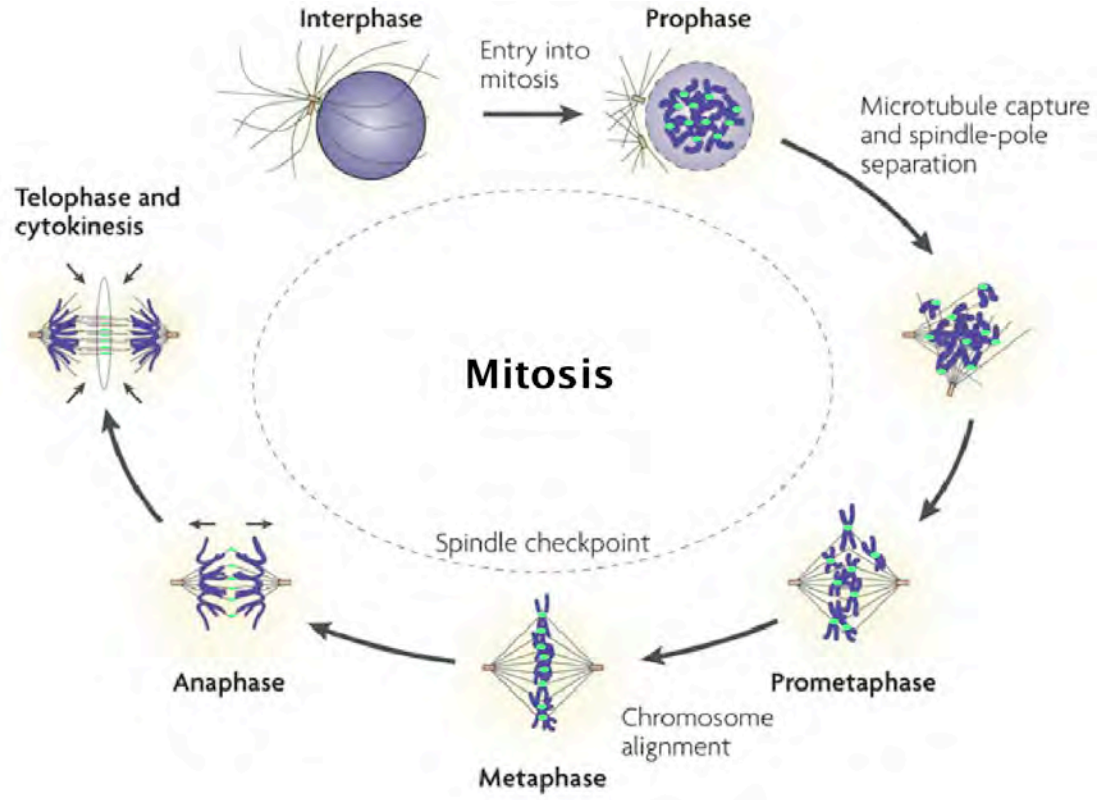

Figure 2.6. **Specific localization of chromosomal passenger complex proteins during mitosis.** CPC members (green) are depicted during the stages of mitosis illustrating microtubule (black) reorganization and chromosome (violet) translocation, for details see text (Figure modified from Jackson *et al.* 2007).

AuroraB is a serine threonine kinase and INCENP is its activating and targeting subunit. The exact functions of Survivin and Borealin are not yet firmly know. However, it seems that Borealin is required for stability of the bipolar mitotic spindle, and Survivin is responsible to recruit all the

other members of the chromosomal passenger complex to the spindle midzone.

Homologues of the "***In**ner **Cen**tromer **P**rotein*" INCENP have been identified in all species from human to yeast. The hallmark of these proteins is the so-called IN-box, a 60-80 amino acid long region that binds to AuroraB in mitosis and is subsequently phosphorylated which in turn enhances AuroraB kinase activity. In higher eukaryotes, inactivation of this gene leads to defects in chromosome congression and failure in cytokinesis.

Substrates of the AuroraB kinase, which is also highly evolutionary conserved, comprise histone H3, CENP-A ("***Cen**tromer **P**rotein **A***"), INCENP and Survivin, with the list of potential and real substrates still expanding. AuroraB inactivation leads to severe phenotypes: Loss of kinetochore attachment to microtubules, exit from mitosis without the completion of anaphase, unattached chromosomes and defects in kinetochore assembly.

Survivin's localization seems to be strictly dependent on INCENP and vice versa. Survivin constitutes an essential gene in most organisms, as mutations induce defects in chromosomal alignments, spindle assembly and cytokinesis.

One role of the complex is the promotion and stabilization of protein recruitment to kinetochores, as it is required for the stable targeting of the checkpoint proteins BUBR1 and MAD2 to unattached kinetochores. In the presence of microtubules, AuroraB is also required for the recruitment of CENP-E, MCAK and dynein to centromeres. The assumption that the passenger complex plays a role in chromosome condensation is still under debate. Disruption of any passenger protein leads to the accumulation of chromosomes that are unable to congress to the metaphase plate. Monopolar attachment, which is the attachment of both kinetochores to microtubules emanating from the same side, is a common cause of aneuploidy in cells. Experimental data suggest that the chromosomal passenger complex is implicated into the correction of these events. The current model states that the activity of the passenger complex responds to spindle tension. A physical separation between AuroraB and its substrates by a stretched bi-oriented centromere could be the trigger for the inactivation of the complex to allow progression in the cycle.

Thus, not only the exact role of passenger proteins in cytokinesis is still under debate, also the detailed contribution of the different components to its function is not yet clarified. Especially the role of Survivin with its proposed dual role as an apoptosis inhibitor and a mitotic effector was controversially discussed. The fact that survivin could be detected as a cytoplasmic and as a nuclear protein in interphase cells of cancer patients, stimulated numerous studies to investigate and to speculate on the functional significance of its dynamic localization, also during mitosis.

2.7 Nucleo-cytoplasmic transport

One defining key feature of eukaryotic cells is their spatial and functional division into the nucleus and the cytoplasm. In contrast to prokaryotes which only possess one cellular compartment, numerous fundamental biological processes can be regulated more sophisticatedly, and thus a much more complex level of intra- and intercellular communication can be achieved. To efficiently control fundamental biological processes like signal transduction, transcription and translation in a time and space dependent manner, the nucleus, comprising most of the cell's genetic material, and the cytoplasm, where protein synthesis takes place, are separated by the nuclear envelope and transport occurs through the nuclear pore complexes. This type of regulation requires the existence of a highly specific and efficient transport machinery for the controlled transport of macromolecules between both compartments. Ordered regulation of bidirectional nucleo-cytoplasmic transport is critical for normal cell function and essential for cellular homeostasis. Deregulation of nucleo-cytoplasmic transport has been observed in many disease conditions. Thus, besides the academic interest in a detailed understanding of the molecular regulation of nucleo-cytoplasmic transport, targeted intervention with transport is now considered also an attractive opportunity for the development of novel therapeutics (see Knauer 2005).

All nucleo-cytoplasmic transport processes take place through the nuclear pores that are embedded in the nuclear envelope (reviewed in Fahrenkrog *et al.* 2004; Pante 2004). Small molecules up to a size of 60 kDa are able to passively diffuse through the pore channels, whereas larger molecules or complexes must be actively transported in an energy-dependent manner (Pante & Kann 2002). This active transport can also occur against a concentration gradient, and is mediated by soluble transport factors, that in turn shuttle between the nucleus and the cytoplasm. Importantly, even molecules that are theoretically small enough for passive diffusion are actively and selectively transported, and often play crucial roles for cellular homeostasis (Görlich & Kutay 1999), since regulated transport appears to be more efficient and more amendable for specific regulation.

The Ran-GTPase cycle

One of these soluble factors, which plays an important role in conferring directionality to nucleo-cytoplasmic transport events, is the small Ras-like GTPase (guanosine-5'-triphosphatase) Ran (Izaurralde *et al.* 1997; Nachury & Weis 1999). Similar to other Ras-like GTPases, Ran occurs in two differently bound states inside the cell (reviewed in Görlich & Kutay 1999; Kuersten *et al.* 2001)(see Fig. 2.6.1). Either it is bound to guanosine triphosphate (GTP), or it is complexed to guanosine diphosphate (GDP). A steep Ran-GTP/Ran-GDP gradient with nuclearly enriched Ran-GTP is generated by the cellular compartmentalization of the regulators of the Ran cycle. Specifically, the guanine-nucleotide exchange factor of Ran (RanGEF or RCC1), which regenerates Ran-GTP, is nuclear and associated with the chromatin (Azuma & Dasso 2000; Dasso 2001). In contrast, the main GTPase-activating protein (RanGAP) and its co-activators, the Ran-binding proteins RanBP1 and RanBP2, which stimulate GTP hydrolysis, localize to the cytoplasm (Dasso 2001). As a direct consequence, nuclear Ran is predominantly bound to GTP, whereas cytoplasmic Ran is immediately converted to a GDP-bound state.

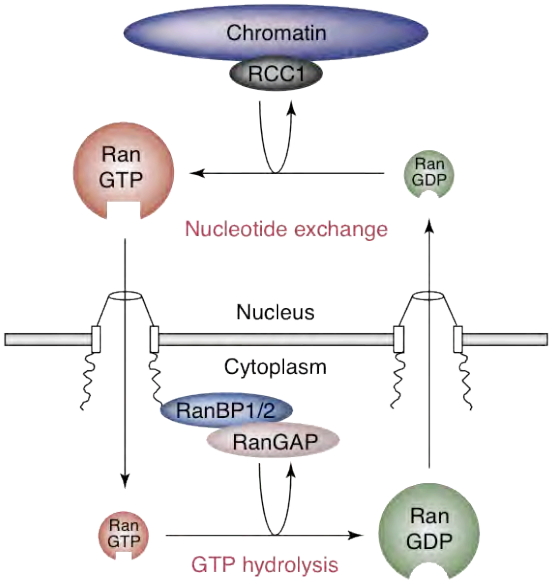

Figure 2.7.1. **The Ran-GTP/GDP cycle.** Figure modified from Kuersten *et al.* 2001. See text for details.

The protein family of karyopherins

Most nucleo-cytoplasmic transport processes are mediated by a group of homologous transport receptors, the karyopherins. They belong to the family of importin-β-like proteins, named after the first characterized member importin-β (Görlich & Laskey 1995). In vertebrates, there exist at least 20 different karyopherins recognizing their various cargoes via intrinsic transport signals (reviewed in Pemberton & Paschal 2005). Depending on the direction of transport, karyopherins are divided into importins und exportins, with the potential to recognize either substrates with an nuclear import/localization (NLS) or nuclear export signal (NES), respectively. Importins bind their cargo in the cytoplasm and transfer it to the nucleus, whereas exportins interact with their substrates in the nucleus and mediate their export to the cytoplasm. All karyopherins consist of a N-terminal Ran-GTP-binding site and a C-terminal domain mediating the interaction with their cargoes. At the same time, the karyopherins can interact with the FG-repeats of the NUPs and thereby enable the translocation of the complexes through the pore. Despite their detailed characterization, the exact molecular mechanism how specificity and activity of the karyopherin-cargo interaction is achieved and controlled is still not completely understood.

Import processes

Protein translocation through the NPC is thought to occur by an essentially similar mechanism for all importin-β related receptors, except for the fact that, in some situations, additional adaptors are required to bridge the cargo/receptor interaction (reviewed in Goldberg 2004; Harel & Forbes 2004; Pemberton & Paschal 2005).

The most-studied pathway is the import of classical NLS-containing proteins. Classical protein import is mediated by one or two clusters of basic amino acids (aa), the simple or classical basic nuclear localization signal, and the bipartite NLS (Kalderon *et al.* 1984; Robbins *et al.* 1991). E.g.,

the simian virus 40 (SV40) large T-antigen harbors a classical NLS, consisting of the aa-sequence PKKKRKV. The import of many nuclear proteins is thought to be mediated by the basic NLS. Both types of classical import signals are recognized by the heterodimeric importin-β/importin-α complex in the cytoplasm. Thereby, importin-α acts as an adaptor between the NLS-bearing protein and importin-β, recognizing and binding the NLS-containing cargo and importin-β in the cytoplasm. Translocation of the complex occurs in the presence of Ran-GDP, and is terminated via binding of Ran-GTP to importin-β in the nucleus, which releases the complex from the NPC and dissociates importin-α from importin-β. Thereafter, the importins are re-exported to the cytoplasm for another round of import.

In higher eukaryotes, several importin-β homologues exist, some of which interestingly show a tissue- and development-specific expression. Additionally, they reveal different binding specificities to NLS-bearing cargo proteins (Nachury *et al.* 1998). This suggests that import processes can be regulated specifically for different tissue types. In addition, there is increasing evidence that active import of cargos containing basic NLSs can be mediated by importin-β in the absence of importin-α (Palmeri & Malim 1999; Strom & Weis 2001).

Export processes

As one hallmark of nucleo-cytoplasmic transport is the bidirectionality, the existing import reactions into the nucleus face a comparable amount of export processes into the cytoplasm. These transport processes in the reverse direction are mediated by exportins, and are regulated in a converse manner (reviewed in Goldberg 2004; Kau *et al.* 2004; Pemberton & Paschal 2005).

The best characterized signal mediating export is the leucine-rich NES, which was first discovered within the Rev-protein of the human immunodeficiency virus type 1 (HIV-1) (Fischer *et al.* 1995). The HIV-1 Rev protein is an essential viral protein responsible for the efficient export of unspliced and partially spliced viral mRNAs required for the production of the viral structural proteins.

Leucine-rich NESs conform to the still loosely defined consensus sequence Ω-x_{2-3}-Ω-x_{2-3}-Ω-x-I/L, (Ω = V,I,L,F,M or W; x is any amino acid) (Fornerod & Ohno 2002; la Cour *et al.* 2004). The presence of regularly spaced, large hydrophobic amino acids such as leucine or isoleucine as well as the spacing itself are critical features of the signals. Leucine-rich NESs have been identified in an increasing number of disease relevant cellular and viral proteins (see Table 2.6.) (Heger *et al.* 1999; Heger *et al.* 2001; la Cour *et al.* 2004), implicated in transcription control, cell cycle control and RNA transport.

Table 2.7. **Examples of viral and vertebrate leucine-rich NESs.**

Protein	NES-sequence
Minute virus of mice (MVM) NS2	**M**TKK**F**–GTL**L**T**I**
Protein kinase inhibitor PKI	**L**ALK**L**–AG**L**D**I**
HIV-1 Rev	**L**–PP**L**–ER**L**T**L**
HTLV-1 Rex	**L**SAQ**L**YSS**L**S**L**
MAP kinase kinase (MAPKK)	**L**QKK**L**–EE**L**E**L**
Adenovirus type 5 E1B-55K	**L**YPE**L**RR**I**L**T**I
Tumor suppressor protein p53	**M**FRE**L**NEA**L**E**L**
Double minute 2 Mdm2	**I**SLS**F**DES**L**A**L**
Inhibitor of NF-kB I-kB	**M**VKE**L**–QE**I**R**L**
Cyclin B1	**L**CQA**F**–SD**V**I**L**
Transcription factor IIIA TFIIIA	**L**–PV**L**–EN**L**T**L**
Signal transducer and activator of transcription STAT1	**L**AAE**F**-RH**L**Q**L**
NES consensus	**Ω**x_{2-3}**Ω**x_{2-3}**Ω**x**L**/**I**

Conserved hydrophobic aa residues reported to be essential for function are marked in bold.

Ω denotes amino acids V,F,M or W; x is any aa.

A major step towards the identification of the export receptor of leucine-rich NESs was the observation that the fungicide antibiotics leptomycin B (LMB) blocks export of Rev (Wolff *et al.* 1997). LMB, a Streptomyces metabolite, inhibits the cellular target protein CRM1 (chromosome region maintenance) by direct binding (Fornerod *et al.* 1997; Fukuda *et al.* 1997). The inhibition of CRM1 by LMB depends on the highly conserved nucleophilic cysteine residue 528 (Cys528), to which LMB covalently binds in a "Michael-type" reaction (see Figure 2.6.2) (Kudo *et al.* 1999). Thus, LMB serves as a potent tool to identify proteins that are exported via the CRM1-pathway.

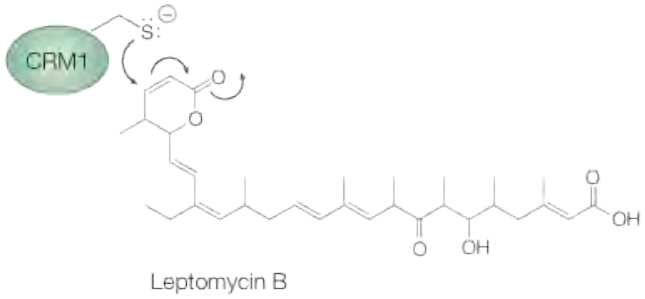

Figure 2.7.2. **Michael-type addition of LMB with the Cys528 of CRM1.** Figure taken from Kau *et al.* 2004.

Mechanistically, CRM1 binds to substrates containing a leucine-rich NES in the nucleus, forming a trimeric complex with Ran-GTP. This complex is then transferred to the cytoplasm by a mechanism involving binding of CRM1 to the NPC. Once in the cytoplasm, GTP hydrolysis results in the dissociation of Ran from the complex, allowing CRM1 to release its cargo. Free CRM1 then reenters the nucleus to bind and export additional cargo molecules.

Although the orchestration of export is still unclear, NESs can be grouped into specific classes according to their activity *in vivo* (Rosorius *et al.* 1999; Heger *et al.* 2001), although most NESs bind to CRM1 with relatively low affinity *in vitro* (reviewed in Kutay & Guttinger 2005). The different activities of the individual NESs can be regulated either by the NES-conformation itself or by additional cofactors favoring the formation of specific NES-CRM1 complexes. Efficient binding of weak NESs to CRM1 in the nucleus was for example suggested to be stimulated by a CRM1-specific cofactor, RanBP3, a nuclear Ran-GTP-binding protein (Englmeier *et al.* 2001; Lindsay *et al.* 2001).

Nuclear transport and mitosis

Regulated nucleo-cytoplasmic transport has a profound impact on the intracellular activity of cell cycle regulators. As mentioned above, the small GTPase Ran plays an important role in diverse cellular processes like mitotic spindle assembly, the regulation of cell cycle progression, and post-mitotic nuclear assembly (Dasso 2002). Strikingly, effectors and components of the nuclear transport machinery are not only active during interphase but play additional crucial roles during mitosis. Mitosis involves a dramatic reorganization of the nucleus, with changes in chromatin structure, assembly of the mitotic spindle, and the breakdown of the nuclear envelope. Recent evidence suggest that the Ran-GTPase/RCC1 system also controls changes in microtubule dynamics and chromatin structure (Arnaoutov & Dasso 2003; Weis 2003; Arnaoutov *et al.* 2005). Generation of Ran-GTP by RCC1 on chromosomes causes the release of so called spindle assembly factors (SAFs) from inhibitory complexes with importins-α and -β that otherwise bind to a nuclear localization sequence (NLS) on a SAF (see Figure 2.8) (Clarke 2005). Interestingly, Ran-GTP can also function through CRM1, which interacts with kinetochores and recruits Ran-BP2 and Ran-GAP1. Thus, through the interactions with leucine-rich nuclear export sequences, proteins are recruited as active complexes to the spindle via CRM1. CRM1, which has originally been defined as a chromosome region maintenance in fission yeast (Fornerod *et al.* 1997), can thereby function as a major nuclear export receptor during interphase and as a regulator during mitosis.

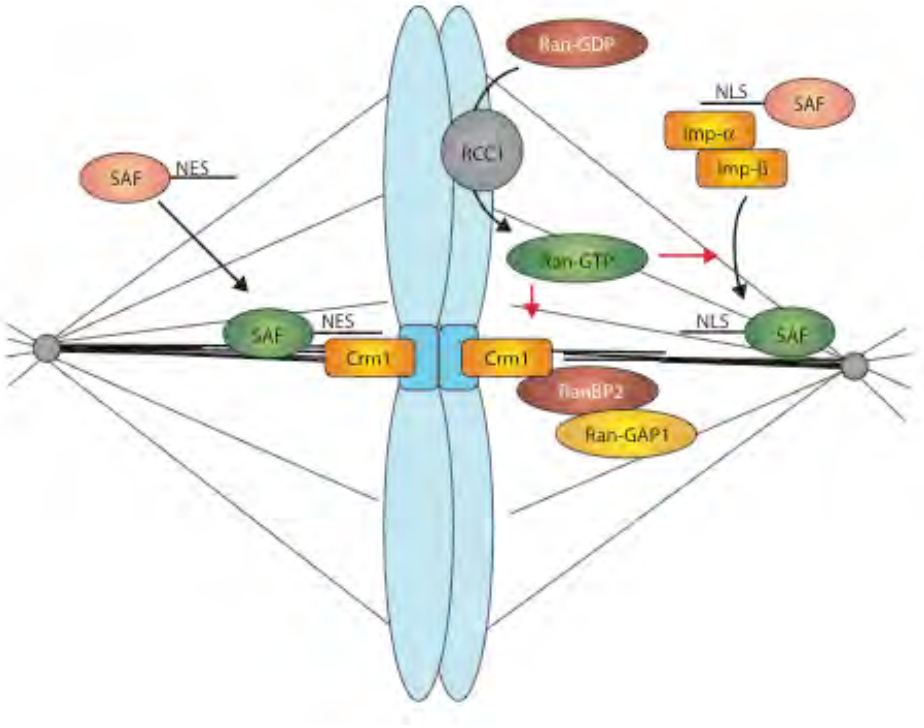

Figure 2.8. **Regulation of multiprotein complexes by Ran-GTP during mitosis.** Figure taken from (Clarke 2005). For further explanations see text.

Regulation of nuclear transport

Nuclear transport is regulated at multiple levels, via a diverse range of mechanisms, and in response to a variety of signals such as hormones, cytokines and growth factors, cell-cycle signals, developmental signals, immune challenge and stress (see Poon & Jans 2005) and references therein). Although several mechanisms have been suggested through which the regulation of nuclear import and export pathways can occur, the complex orchestration and specificity of transport is not yet fully understood. As indicated above, the modulation of the expression level of components of the nuclear transport machinery represents an additional regulatory mechanism. In particular importins appear to be specialized to mediate the nuclear import of particular transport cargoes. Further, it seems likely that importin/cargo complexes or proteins may take specific paths through the NPC by binding to only a subset of FG-repeat-containing NUPs. Thus, the presence or absence of a particular importin or NUP may determine whether specific nuclear import/export cargoes can efficiently enter or exit the nucleus, making the tissue/cell type-specific expression of components of the nuclear transport machinery a critical determinant of the nuclear import efficiency for a given cargo.

Nucleo-cytoplasmic transport and cancerogenesis

Active transport signals have been identified in an increasing number of cellular and viral proteins executing heterogeneous biological functions involving transcription (McBride & Reich 2003; Knauer *et al.* 2005a), apoptosis (Ferrando-May 2005), or cell cycle control (Xu & Massague 2004). Since nucleo-cytoplasmic transport is crucial for normal cell function, defects in this process can also lead to disturbances of the cellular homeostasis, and thereby also contribute to cancer formation (reviewed in Kau *et al.* 2004; Ferrando-May 2005; Poon & Jans 2005). On the other hand, the detailed molecular knowledge of how nucleo-cytoplasmic transport contributes to disease conditions might also be exploited for the formulation of novel therapeutic strategies specifically targeting transport processes.

There are different ways how nucleo-cytoplasmic transport can be deregulated contributing also to malignant transformation. First, modification of the cargo can in turn affect its ability to interact with its cognate transporter. Regulated modifications that affect nuclear transport include phosphorylation, acetylation and ubiquitylation, mostly inducing conformational changes (see Xu & Massague 2004). Constitutive activation of signaling cascades leading to increased phosphorylation and nuclear transport of downstream target molecules, such as the STAT proteins, is observed in a variety of cancers.

On the other hand, dysregulation at the level of the transporters might also lead to cellular transformation. Some karyopherins are expressed only in certain tissues and might transport cargoes only during specific stages of development, or function in a particular cell type (Görlich & Kutay 1999). Components of the nuclear transport machinery also appear to be differentially expressed in transformed cells, with strong proliferative signals leading to the alteration of nuclear import (Kau *et al.* 2004; Sherr 2004). Further examples of an altered nuclear transport machinery in cancer are observed in patients with acute myelogenous leukemia, where chromosomal rearrangements can lead to the fusion of NUPs such as NUP98 or NUP214 with HOXA9 or DEK, respectively. Although these fusion proteins do not assemble into the NPC, their hydrophobic FG-repeat sequences may enable them to bind to transport receptors and modify transport of certain cargoes since overexpression of FG-repeats has been shown to interfere with transport (Kau *et al.* 2004).

Finally, the nuclear pore itself can offer an added level of regulation. The number of functional and/or specific pores may vary depending on the growth state of the cell, which in turn affects the overall permeability of the nucleus.

Thus, modifications to cargo, changes in the nuclear transport machinery and alterations in the NPC itself could markedly alter cellular functions and potentially promote tumorigenesis.

2.8 Targeting nucleo-cytoplasmic transport as a potential therapeutic principle

However, regulated subcellular transport also provides an attractive way to control the activity and stability of regulatory proteins. Deregulation of nucleo-cytoplasmic transport has been observed in many disease conditions, and the cellular transport machinery is also taken advantage of by intracellular parasites, such as viruses. Thus, besides the academic interest in a detailed understanding of the molecular regulation of nucleo-cytoplasmic transport, targeted intervention with transport is now considered also an attractive opportunity for the development of novel therapeutics, and has attracted major interest by academia and industry (reviewed in Kau *et al.* 2004; Pagliaro *et al.* 2004; Knauer 2005).

Drugs that target nucleo-cytoplasmic transport can be envisaged to be active at the different levels described above. However, two important obstacles must be overcome - the problem of specificity for tumor cells versus normal cells, and the difficulty in creating drugs that interfere with protein-protein interactions (PPIs), in contrast to enzyme-substrate binding.

Drugs, which indirectly interfere with nuclear import/export by blocking posttranslational modification of the cargo and thereby inhibiting its ability to interact with its cognate transporter have been described for several proteins. Mostly, these consist of protein kinase inhibitors as exemplified by inhibitors of the PI3K/PTEN/Akt signal transduction pathway, which affected export of the Forkhead family of transcription factors (Kau *et al.* 2003). Although these compounds interfere with nuclear transport of proteins they clearly lack specificity.

So far, no inhibitors have been described directed against components of the stationary nuclear transport machinery. In contrast, the karyopherin transport factors – the karyopherin- proteins, in particular – represent a class of potential targets. Molecular structures of several different karyopherins have been solved, making these proteins potential therapeutic targets, since some of these factors might transport only a defined class of proteins (Kau *et al.* 2004). For example Leptomycin B (LMB), which inhibits CRM1 export activity by covalent binding and prevention of the CRM1-NES interaction, was identified as a HIV-1 inhibitor (Wolff *et al.* 1997) and had also been suggested as a potential anti-cancer drug (Komiyama *et al.* 1985; Vigneri & Wang 2001). However, although LMB clearly inhibits export of the HIV-1 Rev protein or the leukemia inducing Bcr-Abl kinase (Vigneri & Wang 2001), LMB blocks all NES mediated export in the cell, and thus its cellular toxicity will not allow therapeutic applications.

Therefore, protein specific transport inhibitors are urgently needed. Since transport signals can be grouped into specific categories according to their activity *in vivo* (Rosorius *et al.* 1999; Heger *et al.* 2001), these differences may represent an attractive opportunity to selectively interfere with export and the biological functions of proteins by the generation of NES/NLS-specific inhibitors.

Targeting the proteins that are involved in nuclear transport, in addition to the nuclear transport of factors that have been associated with disease, could prove to be a promising approach for controlling cancer-cell growth as well as infectious diseases. In order to efficiently identify nuclear transport and protein-protein interaction small-molecule inhibitors, high-content, cell-based screening assays are urgently required.

3 Aim of the Work

Cancer is one of the leading causes of death in the world, and thereby represents a tremendous burden and future challenge on patients, families and societies. Over the last decades, diagnosis and disease management for most cancer types, including head&neck and colon cancer have improved, but oftentimes long-term survival rates did not. Despite the enthusiasm for optimal cytoreductive surgery and aggressive radio-/chemotherapy with intent to cure, many patients will ultimately not reach this goal. Local-regional relapse after therapy is a major cause of death and has prompted substantial efforts in identifying molecular biomarkers predicting patients at risk for disease recurrence and/or may be serving as novel therapeutic targets.

Current systematic gene expression analysis by our group has identified a variety of genes, which are differentially expressed between primary tumor and the non-neoplastic corresponding normal tissue (Schlingemann *et al.* 2005) and unpublished). Bioinformatic analysis has identified the "inhibitor of apoptosis protein" family member survivin, as one of the most significantly differentially expressed genes. Considerable therapeutic and prognostic interest is focused on this small protein, since its expression has been reported to correlate with reduced tumor cell apoptosis, and increased resistance to cancer therapy (Altieri 2006). Notably, survivin is detected as a cytoplasmic and as a nuclear protein in cancer patients. The molecular mechanisms, by which survivin controls cell division and counteracts apoptosis, have been extensively explored, but are not yet fully understood (Vagnarelli & Earnshaw 2004; Yang *et al.* 2004).

Consequently, the aim of this work was to:

- Investigate the differential expression of wild type (wt) survivin and its splice variants in head&neck and colon cancer cell line models as well as in patient materials.
- Analyse the prognostic potential of wild type (wt) survivin and its splice variants for disease progression and therapy resistance in head&neck and colon cancer patient.
- Investigate the molecular mechanisms, which regulate survivin's dynamic localization and its consequences for the tumor promoting functions of survivin.

To achieve these goals, corrobative experimental methods from the fields of biomedical, molecular and cellular biology in combination with innovative microscopic techniques should be applied.

We expect not only to reveal and understand the clinical relevance of survivin as a significant predictor for disease outcome in cancer patients, but also pave the way for potential novel therapeutic strategies targeting the nodal functions of survivin.

4 RESULTS

4.1 Nucleocytoplasmic Shuttling and the Biological Activity of Mouse Survivin Is Regulated By an Active Nuclear Export Signal

Roland H. Stauber., Uta Rabenhorst, Alexander Rekik, Knut Engels, Carolin Bier, & Shirley K. Knauer

Survivin appears to function as a regulator of cell division and as an apoptosis inhibitor in many species. Human (142 amino acids) and mouse survivin (140 amino acids) are 83,1% identical (Fig. 4.1A). Similar to its human counterpart, two additional murine splice variants, survivin$_{121}$ lacking the coiled-coil domain, and survivin$_{40}$ lacking the IAP repeat as well as the coiled-coil structure, have been described (see Conway *et al.* 2000; Li 2005), Fig. 4.1B). Here, we characterized the nucleocytoplasmic transport of mouse survivin$_{140}$, and its splice variants survivin$_{121}$ and survivin$_{40}$. We show that the dynamic intracellular localization of survivin$_{140}$ is mediated by a Crm1-dependent nuclear export signal (NES) present also in survivin$_{121}$, but absent in survivin$_{40}$. In contrast, neither survivin nor survivin splice variants contain an active nuclear import signal, and seem to enter the nucleus by passive diffusion. The activity of the NES is required for survivin mediated protection against cell death inducing stimuli and influences protein degradation. During mitosis, NES-deficient survivin variants fail to correctly localize to the mitotic machinery and to promote proper cell division. *In vivo* and *in vitro* protein interaction assays show that survivin$_{140}$ and survivin$_{121}$, as well as their export deficient mutants, are able to form homo- as well as heterodimers. The *trans*-dominant negative phenotype observed upon expression of export deficient survivin appears therefore to be mediated by the formation of inactive survivin heterodimers. The survivin-Crm1 axis is essential for the biological activities of murine survivin, and mouse models will allow investigating its functional implications during development and tumorigenesis.

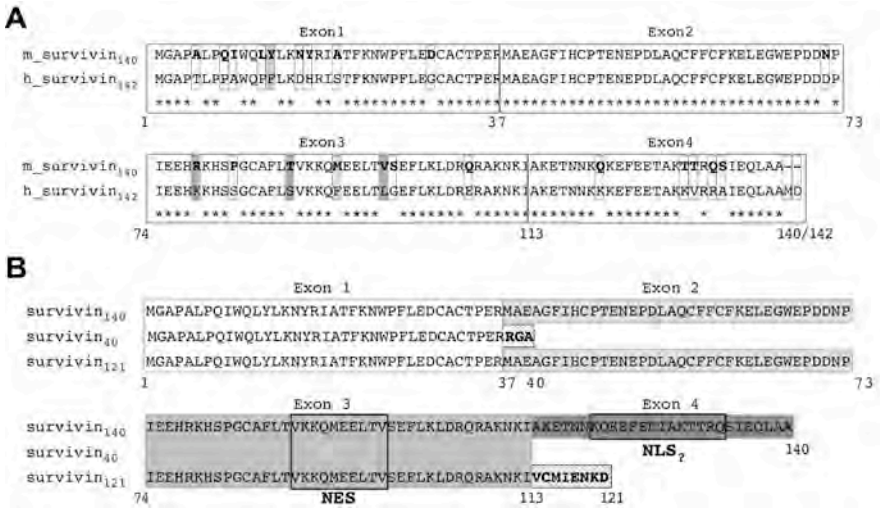

Figure 4.1. **Clustal-based alignment of murine and human survivin (A) and of its murine isoforms (B).** Amino acid positions and exons are indicated. NES: Nuclear export signal. NLS?: potential nuclear localization signal (Figure adapted from Stauber *et al.*, 2006).

4.2 The Survivin-Crm1 interaction mediates targeting of the chromosomal passenger complex to the centromere

Shirley K. Knauer, Carolin Bier, Negusse Habtemichael and Roland H. Stauber

The chromosomal passenger complex (CPC) of Aurora-B, Borealin, INCENP, and Survivin coordinates essential chromosomal and cytoskeletal events during mitosis. Here we show that the nuclear export receptor Crm1 is critically involved in tethering the CPC to the centromere by interacting with a leucine-rich nuclear export signal (NES) (Fig 4.2), evolutionary conserved in all mammalian Survivin proteins. We demonstrate that inhibition of the Crm1/Survivin interaction by treatment with leptomycin B, or by RNAi-mediated Crm1 depletion prevents centromeric targeting of Survivin. Importantly, the genetic inactivation of the Survivin/Crm1 interaction by mutation of the NES affects the correct localization and function of Survivin and the CPC during mitosis. In contrast, CPC assembly appears not to require the Survivin/Crm1 interaction. Our report demonstrates the functional significance of the Crm1/Survivin interface and provides a novel link between the mitotic effector Crm1 and the CPC.

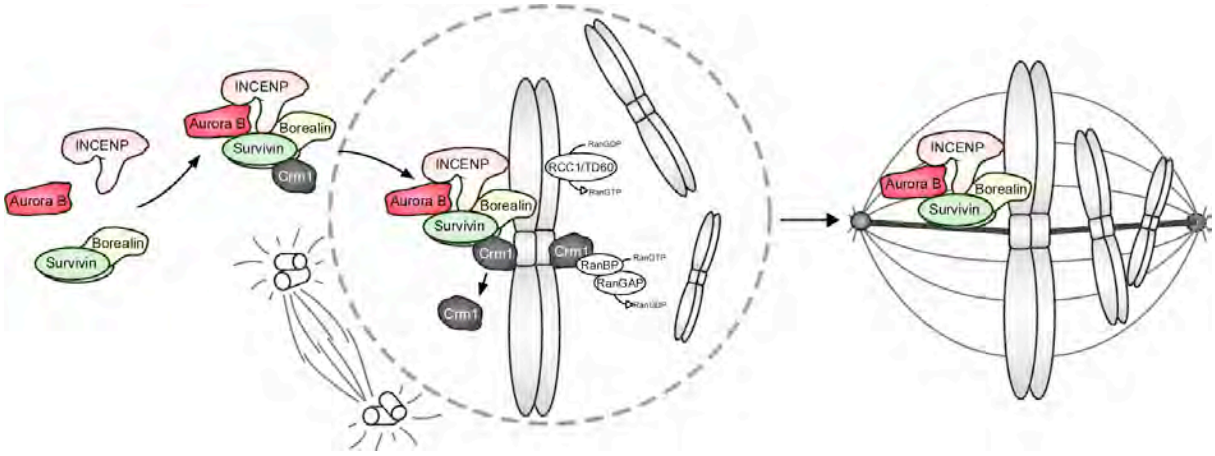

Figure 4.2. **Model for the role of Crm1 in targeting the CPC to the centromere** (see text).

4.3 Dynamic Intracellular Survivin in Oral Squamous Cell Carcinoma – Underlying Molecular Mechanism and Potential as an Early Prognostic Marker.

Knut Engels, Shirley K. Knauer*, Dirk Metzler, Carina Simf, Oliver Struschka, Carolin Bier, Wolf Mann, Adoriàn F. Kovács and Roland H. Stauber**, equal author contribution.

Survivin functions as an apoptosis inhibitor and a regulator of cell division in many tumors. The intracellular localization of survivin in tumors is discussed as a prognostic marker. However, current reports are inconsistent, and the underlying molecular mechanisms are not understood. We examined the localization and prognostic value of nuclear and cytoplasmic survivin in the pre-therapeutic biopsies from 71 oral and oropharyngeal squamous carcinoma (OSCC) patients (Fig. 4.3). Statistical analysis indicated that preferential nuclear versus cytoplasmic survivin is correlated with favourable versus unfavourable disease outcome. Uni- and multivariate analysis showed that in contrast to total survivin expression the difference of nuclear and cytoplasmic survivin is a strong predictor for relapse free survival (p=0.0003). As a potential underlying molecular mechanism, we show in OSCC cell lines that predominantly cytoplasmic survivin mediates protection against chemo- and radiotherapy-induced apoptosis. Importantly, the cytoplasmic localization of survivin is regulated by its nuclear export signal (NES), and export deficient nuclear survivin is not cytoprotective. Our study suggests the difference of cytoplasmic and nuclear survivin as an indicator for survivin activity in tumor cells. Thus, this difference may serve as a predictive marker of outcome in OSCC patients undergoing multimodality therapy. We also propose to pursue the pharmacogenetic interference with survivin's cytoplasmic localization as a potential therapeutic strategy.

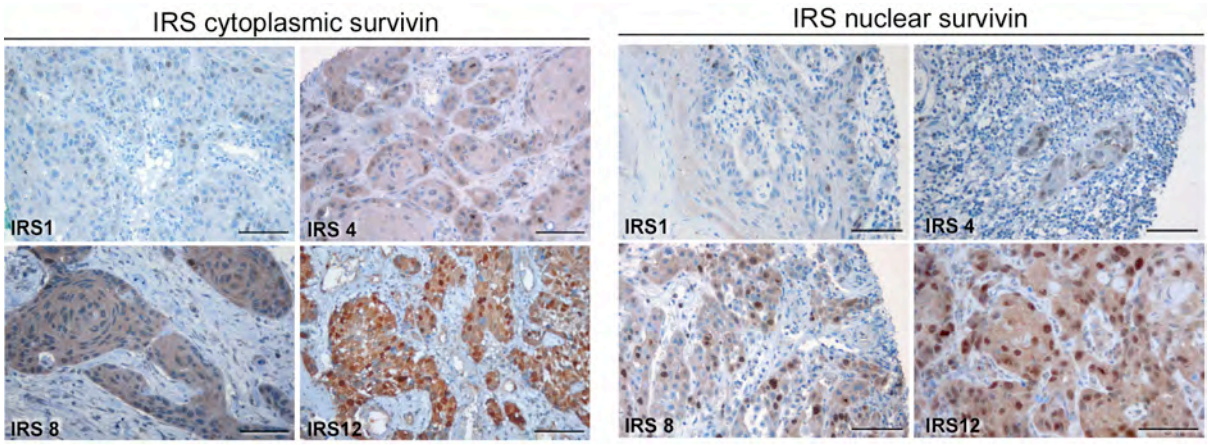

Figure 4.3. **Cytoplasmic and nuclear survivin expression in tumor cells in pre-therapeutic biopsies from OSCC patients.** IHC staining was performed using the α-survivin antibody. Cell nuclei were counterstained with hematoxylin. Representative examples for IRS_{Scyt} (left panel) and for IRS_{Snuc} (right panel) 1, 4, 8, and 12 are shown. Scale bars, 50 µm.

4.4 Nuclear export is essential for the tumor promoting activity of survivin

Shirley K. Knauer, Oliver H. Krämer, Thomas Knösel, Knut Engels, Franz Rödel, Adoriàn F. Kovács, Wolfgang Dietmeier, Ludger Klein-Hitpass, Negusse Habtemichael, Andrea Schweitzer, Jürgen Brieger, Claus Rödel, Wolf Mann, Iver Petersen, Thorsten Heinzel and Roland H. Stauber

Survivin appears to function as an apoptosis inhibitor and a regulator of cell division during development and tumorigenesis. Here, we report the molecular characterization of the nucleocytoplasmic transport of survivin and its potential implications for tumorigenesis. We identified an evolutionary conserved Crm1-dependent nuclear export signal (NES) in survivin (Fig. 4.4). In dividing cells, the NES is essential for tethering survivin and the survivin/Aurora-B kinase complex to the mitotic machinery, which is inevitable for proper cell division. Importantly, export is also required for the cytoprotective activity of survivin, because export deficient survivin fails to protect tumor cells against chemo- and radiotherapy-induced apoptosis. These findings seem to be of clinical relevance since preferential nuclear localization of survivin correlated with enhanced survival in colorectal cancer patients. Targeting survivin's nuclear export by the application of NES-specific antibodies promoted its nuclear accumulation and inhibited its cytoprotective function. We demonstrate that nuclear export is essential for the biological activity of survivin and encourage the identification of molecular decoys to specifically interfere with survivin's nuclear export as potential anticancer therapeutics.

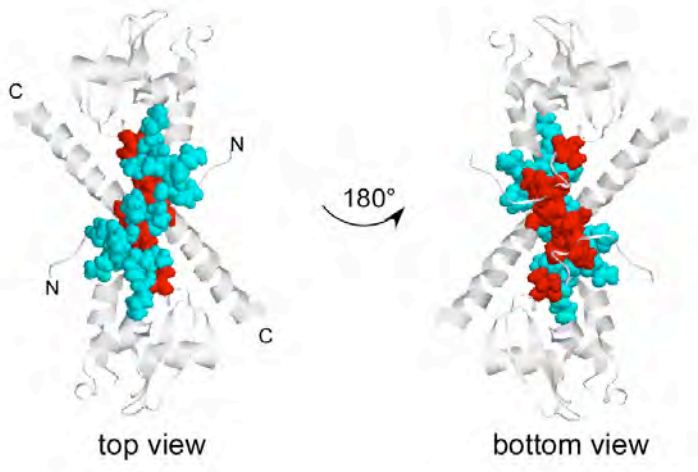

Figure 4.4. **Position of the NES within the NMR structure of human survivin** (PDB 1XOX) (Sun *et al.* 2005). Ribbon representation of the backbone superposition (residues 1-117). Residues 89-98 encompassing the NES are shown in cyan. Amino acids critical for NES activity (aa 89, 93, 96 and 98) are indicated in red.

4.5 Survivin's Dual Role - An Export's View

Shirley K. Knauer, Wolf Mann and Roland H. Stauber

Survivin is proposed to function as a mitotic regulator and an apoptosis inhibitor during development and pathogenesis. As such, survivin has aroused keen interest in disparate areas of basic and translational research. Survivin acts as a subunit of the chromosomal passenger complex (CPC), composed of the mitotic kinase Aurora-B, Borealin and INCENP, and is essential for proper chromosome segregation and cytokinesis. Our recent findings indicate that the nuclear export receptor Crm1 is critically involved in tethering the CPC to the centromere by interacting with a leucine-rich nuclear export signal (NES), evolutionary conserved in all mammalian survivin proteins. In addition, the survivin/Crm1 interaction seems to be required for the cytoprotective activity of survivin, because export deficient survivin fails to protect tumor cells against cancer therapy-induced apoptosis. These findings appear of clinical relevance since preferential nuclear localization of survivin turned out to be a favorable prognostic factor in cancer patients. Besides emphasizing the functional significance of the Crm1/survivin interface (Fig. 4.5), we suggest to exploit the pharmacogenetic interference with survivin's export as a novel strategy to antagonize survivin's activity.

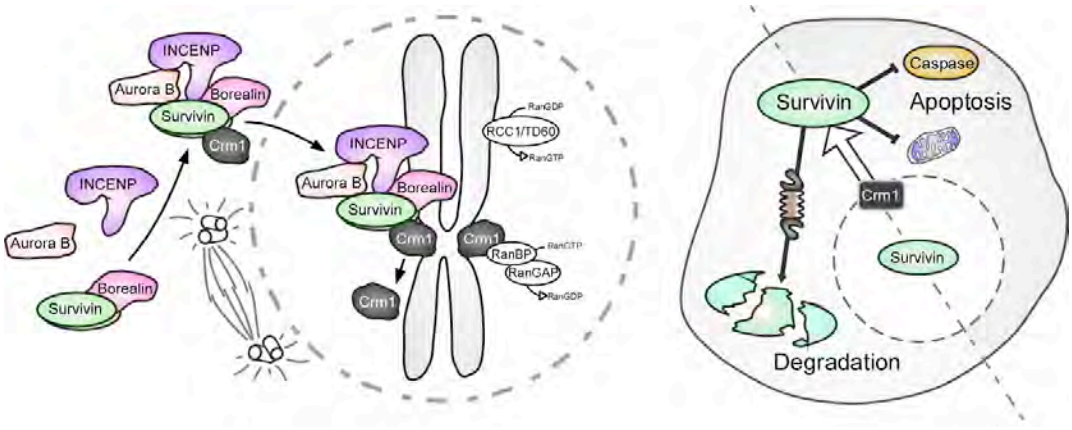

Figure 4.5. **Proposed model how the Crm1/survivin-axis supports the dual activity of survivin.** At the beginning of mitosis, the Crm1/survivin interaction is critically involved in tethering the CPC to the centromere (for details see text). Upon reassembly of the nuclear envelope at the end of mitosis, Crm1 mediates the removal of survivin from the nucleus, which may facilitate proteasomal degradation in the cytoplasm. In interphase cells, nuclear export promotes a high cytoplasmic (and mitochondrial) concentration of survivin to counteract pro-apoptotic stimuli.

4.6 The survivin splice variant survivin-3B is cytoprotective and can function as a chromosomal passenger complex protein

Shirley K. Knauer, Carolin Bier, Peter Schlag, Johannes Fritzmann, Wolfgang Dietmeier, Franz Rödel, Ludger Klein-Hitpass, Adoriàn F. Kovács, Claudia Döring, Martin-Leo Hansmann, Wolf-Karsten Hofmann, Martin Kunkel, Christoph Brochhausen, Knut Engels, Burkhard M. Lippert, Wolf Mann and Roland H. Stauber

Survivin is described as a bifunctional protein inhibiting apoptosis and regulating mitosis. The *survivin* gene on chromosome 17q25 gives also rise to four alternatively spliced transcripts (Li 2005). Although not all variants have been unambiguously shown to be expressed *in vivo*, the transcripts potentially encode proteins of 74 (survivin$_{2\alpha}$), 120 (survivin$_{3B}$), 137 (survivin$_{\Delta Ex3}$), 142 (survivin), or 165 (survivin$_{2B}$) amino acids (aa)(Li 2005)(Figure 4.6). However, the biological functions and contributions to cancer progression of survivin splice variants are controversially discussed. We here show that the intracellular localization of these splice variants depends on a Crm1-dependent nuclear export signal (NES) present in survivin, survivin$_{2B}$ and survivin$_{3B}$, but absent in survivin$_{\Delta Ex3}$ and survivin$_{2\alpha}$ (Fig. 4.6). Survivin isoforms lack an active nuclear import signal and are able to enter the nucleus by passive diffusion. Only survivin$_{3B}$ but none of the other splice variants is cytoprotective and able to efficiently interact with chromosomal passenger complex (CPC) proteins. The NES together with efficient CPC formation is required for the cytoprotective activity of survivin isoforms, as well as for their correct localization and function during cell division. In the tumors from breast, colorectal, head and neck cancer, lymphoma and leukemia patients, survivin and survivin$_{2B}$ were found overexpressed. However, survivin was the predominant form detected, and the other survivin isoforms were only expressed at low levels in tumors. Our data provide a molecular rationale for the localization and activity of survivin variants, and conclude that survivin isoforms are unlikely to modulate survivin *in trans* in cancer patients.

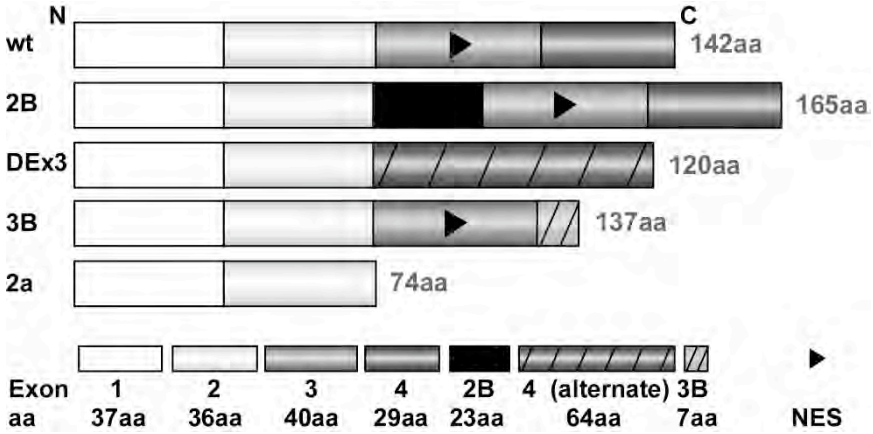

Figure 4.6. **Schematic representation of survivin and its splice variants.** Exons are indicated. NES: Nuclear export signal.

4.7 Dynamic survivin in head and neck cancer: Molecular mechanism and therapeutic potential

Burkhard M. Lippert, Shirley K. Knauer*, Verena Fetz, Wolf Mann and Roland H. Stauber;**, authors contributed equally to this work.

Although disease management of head and neck squamous cell carcinomas (HNSCC) has improved significantly, therapy resistance leading to tumor recurrence still counteracts improvement of long-term survival. Consequently, identification of molecular markers that signal increased risk of treatment failure or that can be exploited by targeted therapy are urgently needed. Survivin is strongly expressed in HNSCC, and its proposed dual role as an apoptosis inhibitor and a mitotic effector positioned survivin in the front line of cancer research. Notably, survivin is detected as a cytoplasmic and as a nuclear protein in HNSCC patients, which stimulated numerous studies to investigate and to speculate on the functional and prognostic significance of its dynamic localization. This review focuses on our current understanding of the molecular mechanisms regulating survivin's intracellular localization (Fig. 4.7) and discusses its potential prognostic and therapeutic relevance for head and neck cancer.

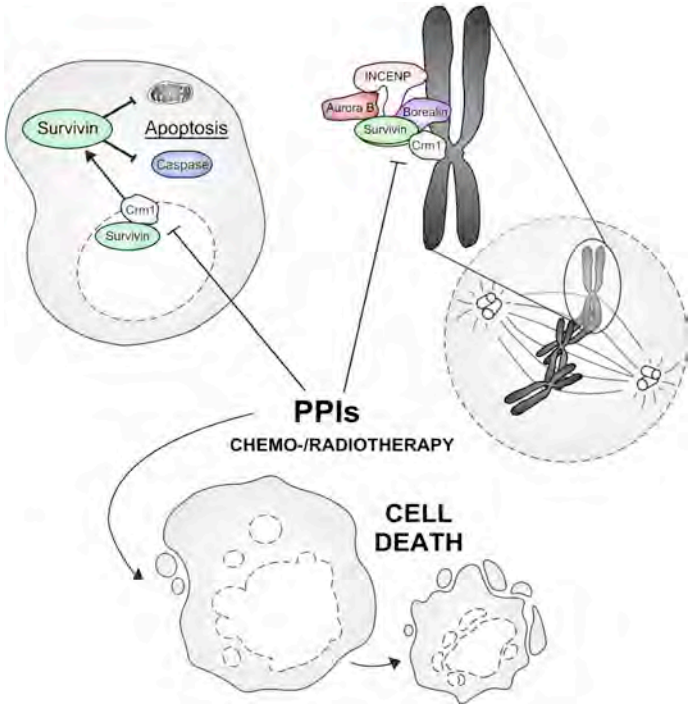

Figure 4.7. **Extended model how the Crm1/survivin-axis supports the dual activity of survivin.** *Left*: In interphase cells, nuclear export promotes a high cytoplasmic concentration of survivin to counteract pro-apoptotic stimuli. *Right*: During mitosis, the Crm1/survivin interaction is critically involved in tethering the CPC to the centromere. Pharmacological targeting of the survivin/Crm1 interaction by protein–protein interaction inhibitors (PPIs) in combination with current chemoradiation treatment protocols may result in increased tumor cell death.

4.8 Nuclear and Cytoplasmic Survivin: Molecular Mechanism, Prognostic, and Therapeutic Potential.

Roland H. Stauber, Wolf Mann and Shirley K. Knauer

Its proposed dual role as an apoptosis inhibitor and a mitotic effector positioned survivin in the front line of cancer research. Notably, survivin is detected as a cytoplasmic and as a nuclear protein in cancer patients, which stimulated numerous studies to investigate and to speculate on the functional and prognostic significance of its dynamic localization. Recent evidence demonstrates that survivin's direct interaction with the nuclear export receptor Crm1 is critically involved in its intracellular localization and cancer relevant functions. Here, we review our current understanding of the Crm1/survivin interface and discuss its potential prognostic and therapeutic relevance.

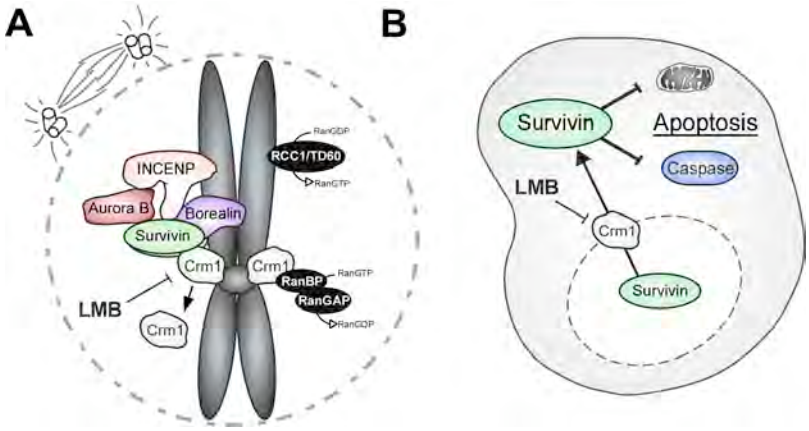

Figure 4.8. **Model how the Crm1/survivin-axis supports the dual activity of survivin.** A. Role of Crm1 in targeting the CPC to the centromere. Borealin is complexed with Survivin, which can bind to Aurora-B kinase and is incorporated into the CP-holocomplex by interacting with INCENP. The NES in Survivin mediates recruitment of Crm1/RanGTP, which guides the CPC to the centromeres by a still unknown mechanism. This process might be catalyzed by the activity of the guanine nucleotide-exchange factor RCC1 or TD60. Hydrolysis of RanGTP, by factors like RanBPs/Ran-GAP1, may facilitate the release of Crm1 and deposition of the CPC at the inner centromere. B. In interphase cells, nuclear export promotes a high cytoplasmic (and mitochondrial) concentration of survivin to counteract pro-apoptotic stimuli.

5 ACHIEVEMENTS OF THIS WORK AND OUTLOOK

As the efficacy of mainstay cancer therapies like chemo and radiotherapy has reached a plateau in the treatment of many cancers, there is a sense of urgency that improvements must now come from fresh approaches. 'Target-oriented' approaches are aimed specifically at proteins that are 'conceptually' important for tumor maintenance, and tailored to eliminate tumor cells while sparing normal tissues. However, the overwhelming majority of cancers defies single-molecule-directed therapy and quickly become resistant. In contrast, drugs targeting "nodal proteins" that are involved in multiple signaling mechanisms of tumor maintenance might go beyond single-molecule antagonists. Such "pathway inhibitors" may be able to globally affect multiple signaling circuits in tumor cells, regardless of complexity, heterogeneity, or genetic make-up.

The role of survivin as a potential "nodal cancer protein", orchestrating extensive, and potentially "tumor-specific", signaling networks is supported by many reports (Fig. 5).

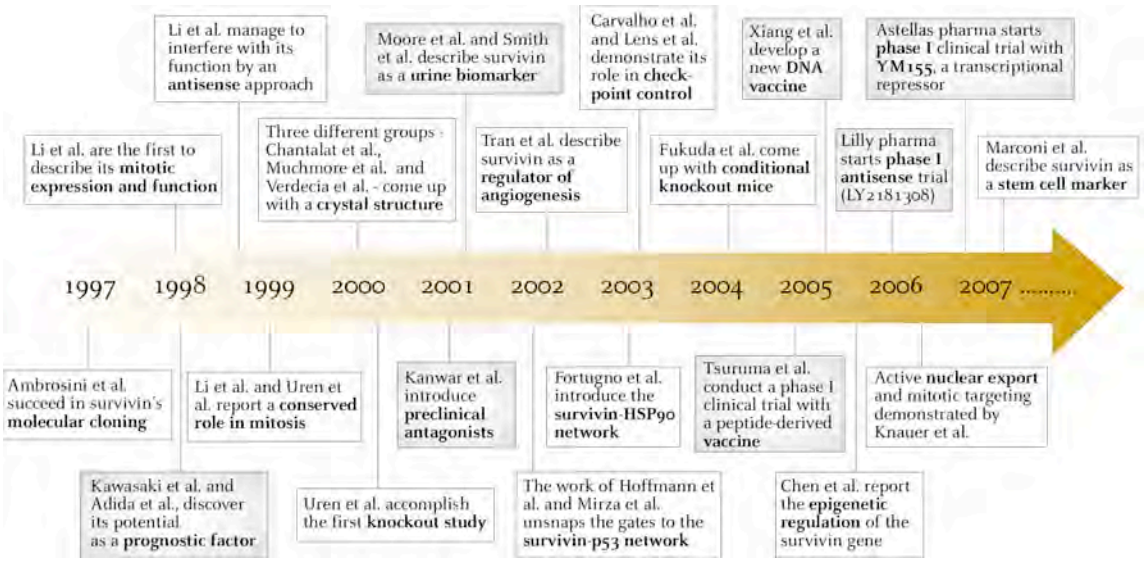

Figure 5. **Timeline of 10 years survivin research.** Grey boxes indicate clinically relevant findings.

However, the molecular mechanisms, by which survivin controls cell division and counteracts apoptosis, have been extensively explored, but are not yet fully understood.

The professional dissertation entitled "*Dynamic Localisation of the tumor-relevant Protein Survivin: Molecular mechanisms, therapeutic and prognostic potential*" aimed to dissect the regulation of survivin's dynamic localisation and its consequences for the tumor promoting functions of survivin in cell culture models and cancer patients.

We here provided evidence that survivin, which we found overexpressed in several tumor entities, is actively exported from the nucleus to the cytoplasm (Knauer *et al.* 2006). Our data argue against the previous assumption that nuclear and cytoplasmic survivin represent two pools with distinct biological activities (Knauer *et al.* 2007c). Rather, survivin is able shuttle between the

nucleus and the cytoplasm, and Crm1's function as an export receptor creates a cytoplasmic survivin concentration gradient counteracted by passive diffusion. A pronounced cytoplasmic survivin localization may promote survivin's cytoprotective activity by facilitating the interplay with the apoptotic machinery. During mitosis, the Crm1/survivin interaction is critically involved in tethering the CPC to the centromeres and thus, ensures proper chromosome segregation (see Fig. 4.5 and 4.7).

Our hypothesis that paramount cytoplasmic survivin represents "cytoprotective survivin" whereas nuclear survivin signals "impaired survivin function" is further supported by clinical data. In head&neck and colon cancer patients, preferential nuclear survivin in tumor cells correlated with favorable disease outcome, whereas high cytoplasmic survivin was associated with poor survival (Engels *et al.* 2007; Lippert *et al.* 2007). Still, the exact molecular mechanisms how survivin displays a predominant nuclear localization in some tumors in contrast to others are not yet resolved. Mutations in the survivin NES, inhibition of the nuclear transport machinery or enhanced binding to overexpressed nuclear survivin interaction partners may contribute to the pronounced nuclear localization of survivin.

In addition, the finding that the presence of a nuclear export signal in the survivin splice variants is also critical for their biological activity further supports the relevance of the survivin/Crm1-axis. Since we found that wt survivin was the predominant and mostly tumor promoting form detectable in different tumor entities, diagnostic efforts as well as therapeutic targeting strategies should focus on wt survivin.

Because of its role as a potential "nodal cancer protein" intersecting multiple cellular networks, survivin is currently vigorously pursued as a cancer drug target by various strategies, ranging from immuno- and gene-therapeutic approaches to the application of small-molecule antagonists (Altieri 2006). Despite the fact that survivin is not a traditional drug target — that is, it is not an enzyme or a cell-surface molecule — our results indicate that molecular decoys selectively targeting the nuclear export of survivin might be of therapeutic relevance. To date, only unspecific export inhibitors inactivating Crm1 (e.g., LMB) have been identified. Although such compounds have been proposed for anticancer therapy (Vigneri & Wang 2001), they can not be used in therapeutic applications due to their toxic side effects by blocking all Crm1-mediated processes (Knauer *et al.* 2005b). To aid in the identification of protein specific transport inhibitors, the three-dimensional structure of survivin (Sun *et al.* 2005) together with cell based translocation- (Knauer *et al.* 2005b) and protein interaction-assays (Knauer & Stauber 2005) have to be exploited for inhibitor identification and design. Clearly, it will be imperative to determine whether a specific pharmacological inhibition of the survivin/Crm1 interface by protein–protein interaction inhibitors (PPIs) can be achieved also in preclinical *in vitro* and animal models. In case of efficacy, early clinical trials should be conducted in cancers that are most likely to respond. Since the molecular significance of the survivin/Crm1 interface has been shown in head&neck and colon cancer cell lines as well as in patients, these tumor entities represent important model systems. Whether the 10th anniversary of survivin will be remembered as the start of a survivin-tailored "*NO GO! - for Cancer Cells*" is the challenge for the future.

Fischer, U., Huber, J., Boelens, W.C., Mattaj, I.W. and Luhrmann, R. (1995). "The Hiv-1 Rev Activation Domain Is a Nuclear Export Signal That Accesses an Export Pathway Used by Specific Cellular Rnas." Cell 82(3): 475-83.

Fornerod, M. and Ohno, M. (2002). "Exportin-Mediated Nuclear Export of Proteins and Ribonucleoproteins." Results Probl Cell Differ 35: 67-91.

Fornerod, M., Ohno, M., Yoshida, M. and Mattaj, I.W. (1997). "Crm1 Is an Export Receptor for Leucine-Rich Nuclear Export Signals." Cell 90: 1051-60.

Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M. and Nishida, E. (1997). "Crm1 Is Responsible for Intracellular Transport Mediated by the Nuclear Export Signal." Nature 390(6657): 308-11.

Gerl, R. and Vaux, D.L. (2005). "Apoptosis in the Development and Treatment of Cancer." Carcinogenesis 26(2): 263-70.

Goldberg, M. (2004). "Import and Export at the Nuclear Envelope." Symp Soc Exp Biol(56): 115-33.

Görlich, D. and Kutay, U. (1999). "Transport between the Cell Nucleus and the Cytoplasm." Annu. Rev. Cell Dev. Biol. 15: 607-60.

Görlich, D. and Laskey, R.A. (1995). "Roles of Importin in Nuclear Protein Import." Cold Spring Harb Symp Quant Biol 60: 695-9.

Hanahan, D. and Weinberg, R.A. (2000). "The Hallmarks of Cancer." Cell 100(1): 57-70.

Harel, A. and Forbes, D.J. (2004). "Importin Beta: Conducting a Much Larger Cellular Symphony." Mol Cell 16(3): 319-30.

Harper, J.V. and Brooks, G. (2005). "The Mammalian Cell Cycle: An Overview." Methods Mol Biol 296: 113-53.

Heger, P., Lohmaier, J., Schneider, G., Schweimer, K. and Stauber, R.H. (2001). "Qualitative Highly Divergent Nuclear Export Signals Can Regulate Export by the Competition for Transport Cofactors in Vivo." Traffic 2(8): 544-55.

Heger, P., Rosorius, O., Hauber, J. and Stauber, R.H. (1999). "Titration of Cellular Export Factors, but Not Heteromultimerization, Is the Molecular Mechanism of Trans-Dominant Htlv-1 Rex Mutants." Oncogene 18(28): 4080-90.

Howard, A. and Pelc, S.R. (1951). "Synthesis of Nucleoprotein in Bean Root Cells." Nature 167(4250): 599-600.

Igney, F.H. and Krammer, P.H. (2002). "Death and Anti-Death: Tumour Resistance to Apoptosis." Nat Rev Cancer 2(4): 277-88.

Izaurralde, E., Kutay, U., von Kobbe, C., Mattaj, I.W. and Gorlich, D. (1997). "The Asymmetric Distribution of the Constituents of the Ran System Is Essential for Transport into and out of the Nucleus." Embo J 16(21): 6535-47.

Jackson, J.R., Patrick, D.R., Dar, M.M. and Huang, P.S. (2007). "Targeted Anti-Mitotic Therapies: Can We Improve on Tubulin Agents?" Nat Rev Cancer 7(2): 107-17.

Jesenberger, V. and Jentsch, S. (2002). "Deadly Encounter: Ubiquitin Meets Apoptosis." Nat Rev Mol Cell Biol 3(2): 112-21.

Kalderon, D., Richardson, W.D., Markham, A.F. and Smith, A.E. (1984). "Sequence Requirements for Nuclear Location of Simian Virus 40 Large-T Antigen." Nature 311(5981): 33-8.

Kau, T.R., Schroeder, F., Ramaswamy, S., Wojciechowski, C.L., Zhao, J.J., Roberts, T.M., Clardy, J., Sellers, W.R. and Silver, P.A. (2003). "A Chemical Genetic Screen Identifies Inhibitors of Regulated Nuclear Export of a Forkhead Transcription Factor in Pten-Deficient Tumor Cells." Cancer Cell 4(6): 463-76.

Kau, T.R., Way, J.C. and Silver, P.A. (2004). "Nuclear Transport and Cancer: From Mechanism to Intervention." Nat Rev Cancer 4(2): 106-17.

Kawasaki, H., Altieri, D.C., Lu, C.D., Toyoda, M., Tenjo, T. and Tanigawa, N. (1998). "Inhibition of Apoptosis by Survivin Predicts Shorter Survival Rates in Colorectal Cancer." Cancer Res 58(22): 5071-4.

Knauer, S.K. (2005). Untersuchungen Zur Bedeutung des Kern-Zytoplasma Transports für die biologische Funktion zellulärer Proteine - Investigations on the Significance of Nucleo-Cytoplasmic Transport for the Biological Function of Cellular Proteins. Georg-Speyer-Haus, Frankfurt. Frankfurt, Johann Wolfgang Goethe-Universität, Frankfurt am Main: 1-221.

Knauer, S.K., Bier, C., Habtemichael, N. and Stauber, R.H. (2006). "The Survivin-Crm1 Interaction Is Essential for Chromosomal Passenger Complex Localization and Function." EMBO Rep 7(12): 1259-65.

Knauer, S.K., Bier, C., Schlag, P., Fritzmann, J., Dietmaier, W., Rodel, F., Klein-Hitpass, L., Kovacs, A.F., Döring, C., Hansmann, M.-L., Hofmann, W.K., Kunkel, M., Brochhausen, C., Engels, K., Lippert, B., Mann, W. and Stauber, R.H. (2007a). "The Survivin Isoform Survivin-3b Is Cytoprotective and Can Function as a Chromosomal Passenger Complex Protein." Cell Cycle 6(12): 553-62.

Knauer, S.K., Carra, G. and Stauber, R.H. (2005a). "Nuclear Export Is Evolutionarily Conserved in Cvc Paired-Like Homeobox Proteins and Influences Protein Stability, Transcriptional Activation, and Extracellular Secretion." Mol. Cell. Biol. 25(7): 2573–82.

Knauer, S.K., Kramer, O.H., Knosel, T., Engels, K., Rodel, F., Kovacs, A.F., Dietmaier, W., Klein-Hitpass, L., Habtemichael, N., Schweitzer, A., Brieger, J., Rodel, C., Mann, W., Petersen, I., Heinzel, T. and Stauber, R.H. (2007b). "Nuclear Export Is Essential for the Tumor-Promoting Activity of Survivin." Faseb J 21(1): 207-16.

Knauer, S.K., Mann, W. and Stauber, R.H. (2007c). "Survivin's Dual Role: An Export's View." Cell Cycle 6(5): 518-21.

Knauer, S.K., Moodt, S., Berg, T., Liebel, U., Pepperkok, R. and Stauber, R.H. (2005b). "Translocation Biosensors to Study Signal-Specific Nucleo-Cytoplasmic Transport, Protease Activity and Protein-Protein Interactions." Traffic 6(7): 594-606.

Knauer, S.K. and Stauber, R.H. (2005). "Development of an Autofluorescent Translocation Biosensor System to Investigate Protein-Protein Interactions in Living Cells." Anal Chem 77(15): 4815-20.

Komiyama, K., Okada, K., Tomisaka, S., Umezawa, I., Hamamoto, T. and Beppu, T. (1985). "Antitumor Activity of Leptomycin B." J Antibiot (Tokyo) 38(3): 427-9.

Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E.P., Wolff, B., Yoshida, M. and Horinouchi, S. (1999). "Leptomycin B Inactivates Crm1/Exportin 1 by Covalent Modification at a Cysteine Residue in the Central Conserved Region." Proc Natl Acad Sci U S A 96(16): 9112-7.

Kuersten, S., Ohno, M. and Mattaj, I.W. (2001). "Nucleocytoplasmic Transport: Ran, Beta and Beyond." Trends Cell Biol 11(12): 497-503.

Kutay, U. and Guttinger, S. (2005). "Leucine-Rich Nuclear-Export Signals: Born to Be Weak." Trends Cell Biol 15(3): 121-4.

la Cour, T., Kiemer, L., Molgaard, A., Gupta, R., Skriver, K. and Brunak, S. (2004). "Analysis and Prediction of Leucine-Rich Nuclear Export Signals." Protein Eng Des Sel 17(6): 527-36.

Lens, S.M., Vader, G. and Medema, R.H. (2006). "The Case for Survivin as Mitotic Regulator." Curr Opin Cell Biol 18(6): 616-22.

Li, F. (2005). "Role of Survivin and Its Splice Variants in Tumorigenesis." Br J Cancer 92(2): 212-6.

Li, F. (2003). "Survivin Study: What Is the Next Wave?" J Cell Physiol 197(1): 8-29.

Li, F., Ackermann, E.J., Bennett, C.F., Rothermel, A.L., Plescia, J., Tognin, S., Villa, A., Marchisio, P.C. and Altieri, D.C. (1999). "Pleiotropic Cell-Division Defects and Apoptosis Induced by Interference with Survivin Function." Nat Cell Biol 1(8): 461-6.

Li, F. and Altieri, D.C. (1999). "The Cancer Antiapoptosis Mouse Survivin Gene: Characterization of Locus and Transcriptional Requirements of Basal and Cell Cycle-Dependent Expression." Cancer Res 59(13): 3143-51.

Li, F., Ambrosini, G., Chu, E.Y., Plescia, J., Tognin, S., Marchisio, P.C. and Altieri, D.C. (1998). "Control of Apoptosis and Mitotic Spindle Checkpoint by Survivin." Nature 396(6711): 580-4.

Lindsay, M.E., Holaska, J.M., Welch, K., Paschal, B.M. and Macara, I.G. (2001). "Ran-Binding Protein 3 Is a Cofactor for Crm1-Mediated Nuclear Protein Export." J Cell Biol 153(7): 1391-402.

Lippert, B., Knauer, S.K., Fetz, V., Mann, W. and Stauber, R.H. (2007). "Dynamic Survivin in Head and Neck Cancer: Molecular Mechanism and Therapeutic Potential." Int J Cancer (in press).

Liston, P., Fong, W.G. and Korneluk, R.G. (2003). "The Inhibitors of Apoptosis: There Is More to Life Than Bcl2." Oncogene 22(53): 8568-80.

McBride, K.M. and Reich, N.C. (2003). "The Ins and Outs of Stat1 Nuclear Transport." Sci STKE 2003(195): RE13.

Meier, P., Finch, A. and Evan, G. (2000). "Apoptosis in Development." Nature 407(6805): 796-801.

Moore, M. (2001). "Urine Detection of Survivin and Diagnosis of Bladder Cancer." J Insur Med 33(2): 202-3.

Muchmore, S.W., Chen, J., Jakob, C., Zakula, D., Matayoshi, E.D., Wu, W., Zhang, H., Li, F., Ng, S.C. and Altieri, D.C. (2000). "Crystal Structure and Mutagenic Analysis of the Inhibitor-of-Apoptosis Protein Survivin." Mol Cell 6(1): 173-82.

Nachury, M.V., Ryder, U.W., Lamond, A.I. and Weis, K. (1998). "Cloning and Characterization of Hsrp1 Gamma, a Tissue-Specific Nuclear Transport Factor." Proc Natl Acad Sci U S A 95(2): 582-7.

Nachury, M.V. and Weis, K. (1999). "The Direction of Transport through the Nuclear Pore Can Be Inverted." Proc Natl Acad Sci U S A 96(17): 9622-7.

Pagliaro, L., Felding, J., Audouze, K., Nielsen, S.J., Terry, R.B., Krog-Jensen, C. and Butcher, S. (2004). "Emerging Classes of Protein-Protein Interaction Inhibitors and New Tools for Their Development." Curr Opin Chem Biol 8(4): 442-9.

Palmeri, D. and Malim, M.H. (1999). "Importin Beta Can Mediate the Nuclear Import of an Arginine-Rich Nuclear Localization Signal in the Absence of Importin Alpha." Mol Cell Biol 19(2): 1218-25.

Pante, N. (2004). "Nuclear Pore Complex Structure: Unplugged and Dynamic Pores." Dev Cell 7(6): 780-1.

Pante, N. and Kann, M. (2002). "Nuclear Pore Complex Is Able to Transport Macromolecules with Diameters of About 39 Nm." Mol Biol Cell 13(2): 425-34.

Pemberton, L.F. and Paschal, B.M. (2005). "Mechanisms of Receptor-Mediated Nuclear Import and Nuclear Export." Traffic 6(3): 187-98.

Poon, I.K. and Jans, D.A. (2005). "Regulation of Nuclear Transport: Central Role in Development and Transformation?" Traffic 6(3): 173-86.

Reed, J.C. and Wilson, D.B. (2003). "Cancer Immunotherapy Targeting Survivin: Commentary Re: V. Pisarev Et Al., Full-Length Dominant-Negative Survivin for Cancer Immunotherapy. Clin. Cancer Res., 9:6523-6533, 2003." Clin Cancer Res 9(17): 6310-5.

Robbins, J., Dilworth, S.M., Laskey, R.A. and Dingwall, C. (1991). "Two Interdependent Basic Domains in Nucleoplasmin Nuclear Targeting Sequence: Identification of a Class of Bipartite Nuclear Targeting Sequence." Cell 64(3): 615-23.

Rosorius, O., Heger, P., Stelz, G., Hirschmann, N., Hauber, J. and Stauber, R.H. (1999). "Direct Observation of Nucleocytoplasmic Transport by Microinjection of Gfp-Tagged Proteins in Living Cells." Biotechniques 27(2): 350-5.

Roy, N., Deveraux, Q.L., Takahashi, R., Salvesen, G.S. and Reed, J.C. (1997). "The C-Iap-1 and C-Iap-2 Proteins Are Direct Inhibitors of Specific Caspases." Embo J 16(23): 6914-25.

Salvesen, G.S. and Duckett, C.S. (2002). "Iap Proteins: Blocking the Road to Death's Door." Nat Rev Mol Cell Biol 3(6): 401-10.

Schimmer, A.D., Welsh, K., Pinilla, C., Wang, Z., Krajewska, M., Bonneau, M.J., Pedersen, I.M., Kitada, S., Scott, F.L., Bailly-Maitre, B., Glinsky, G., Scudiero, D., Sausville, E., Salvesen, G., Nefzi, A., Ostresh, J.M., Houghten, R.A. and Reed, J.C. (2004). "Small-Molecule Antagonists of Apoptosis Suppressor Xiap Exhibit Broad Antitumor Activity." Cancer Cell 5(1): 25-35.

Schlingemann, J., Habtemichael, N., Ittrich, C., Toedt, G., Kramer, H., Hambek, M., Knecht, R., Lichter, P., Stauber, R.H. and Hahn, M. (2005). "Patient-Based Cross-Platform Comparison of Oligonucleotide Microarray Expression Profiles." Lab. Investigation 85: 1024-39.

Sherr, C.J. (2004). "Principles of Tumor Suppression." Cell 116(2): 235-46.

Smith, S.D., Wheeler, M.A., Plescia, J., Colberg, J.W., Weiss, R.M. and Altieri, D.C. (2001). "Urine Detection of Survivin and Diagnosis of Bladder Cancer." Jama 285(3): 324-8.

Stauber, R.H., Mann, W. and Knauer, S.K. (2007). "Nuclear and Cytoplasmic Survivin: Molecular Mechanism, Prognostic, and Therapeutic Potential." Cancer Research 67(13): 5579-83.

Stauber, R.H., Rabenhorst, U., Rekik, A., Engels, K., Bier, C. and Knauer, S.K. (2006). "Nucleocytoplasmic Shuttling and the Biological Activity of Mouse Survivin Are Regulated by an Active Nuclear Export Signal." Traffic 7(11): 1461-72.

Strom, A.C. and Weis, K. (2001). "Importin-Beta-Like Nuclear Transport Receptors." Genome Biol 2(6): REVIEWS3008.

Sun, C., Nettesheim, D., Liu, Z. and Olejniczak, E.T. (2005). "Solution Structure of Human Survivin and Its Binding Interface with Smac/Diablo." Biochemistry 44(1): 11-7.

Takahashi, R., Deveraux, Q., Tamm, I., Welsh, K., Assa-Munt, N., Salvesen, G.S. and Reed, J.C. (1998). "A Single Bir Domain of Xiap Sufficient for Inhibiting Caspases." J Biol Chem 273(14): 7787-90.

Uren, A.G., Beilharz, T., O'Connell, M.J., Bugg, S.J., van Driel, R., Vaux, D.L. and Lithgow, T. (1999). "Role for Yeast Inhibitor of Apoptosis (Iap)-Like Proteins in Cell Division." Proc Natl Acad Sci U S A 96(18): 10170-5.

Vagnarelli, P. and Earnshaw, W.C. (2004). "Chromosomal Passengers: The Four-Dimensional Regulation of Mitotic Events." Chromosoma 113(5): 211-22.

Verdecia, M.A., Huang, H., Dutil, E., Kaiser, D.A., Hunter, T. and Noel, J.P. (2000). "Structure of the Human Anti-Apoptotic Protein Survivin Reveals a Dimeric Arrangement." Nat Struct Biol 7(7): 602-8.

Vigneri, P. and Wang, J.Y. (2001). "Induction of Apoptosis in Chronic Myelogenous Leukemia Cells through Nuclear Entrapment of Bcr-Abl Tyrosine Kinase." Nat Med 7(2): 228-34.

Vogelstein, B. and Kinzler, K.W. (2004). "Cancer Genes and the Pathways They Control." Nat Med 10(8): 789-

Weis, K. (2003). "Regulating Access to the Genome: Nucleocytoplasmic Transport Throughout the Cell Cycle." Cell 112(4): 441-51.

Wolff, B., Sanglier, J.J. and Wang, Y. (1997). "Leptomycin B Is an Inhibitor of Nuclear Export: Inhibition of Nucleo-Cytoplasmic Translocation of the Human Immunodeficiency Virus Type 1 (Hiv-1) Rev Protein and Rev-Dependent Mrna." Chem Biol 4(2): 139-47.

Xu, L. and Massague, J. (2004). "Nucleocytoplasmic Shuttling of Signal Transducers." Nat Rev Mol Cell Biol 5(3): 209-19.

Yang, D., Welm, A. and Bishop, J.M. (2004). "Cell Division and Cell Survival in the Absence of Survivin." Proc Natl Acad Sci U S A 101(42): 15100-5.

6 References

Adida, C., Crotty, P.L., McGrath, J., Berrebi, D., Diebold, J. and Altieri, D.C. (1998). "Developmentally Regulated Expression of the Novel Cancer Anti-Apoptosis Gene Survivin in Human and Mouse Differentiation." Am J Pathol 152(1): 43-9.

Altieri, D.C. (2003a). "Survivin and Apoptosis Control." Adv Cancer Res 88: 31-52.

Altieri, D.C. (2003b). "Blocking Survivin to Kill Cancer Cells." Methods Mol Biol 223: 533-42.

Altieri, D.C. (2003c). "Validating Survivin as a Cancer Therapeutic Target." Nat Rev Cancer 3(1): 46-54.

Altieri, D.C. (2004). "Molecular Circuits of Apoptosis Regulation and Cell Division Control: The Survivin Paradigm." J Cell Biochem 92(4): 656-63.

Altieri, D.C. (2006). "Targeted Therapy by Disabling Crossroad Signaling Networks: The Survivin Paradigm." Mol Cancer Ther 5(3): 478-82.

Ambrosini, G., Adida, C. and Altieri, D.C. (1997). "A Novel Anti-Apoptosis Gene, Survivin, Expressed in Cancer and Lymphoma." Nat Med 3(8): 917-21.

Arnaoutov, A., Azuma, Y., Ribbeck, K., Joseph, J., Boyarchuk, Y., Karpova, T., McNally, J. and Dasso, M. (2005). "Crm1 Is a Mitotic Effector of Ran-Gtp in Somatic Cells." Nat Cell Biol 7(6): 626-32.

Arnaoutov, A. and Dasso, M. (2003). "The Ran Gtpase Regulates Kinetochore Function." Dev Cell 5(1): 99-111.

Azuma, Y. and Dasso, M. (2000). "The Role of Ran in Nuclear Function." Curr Opin Cell Biol 12(3): 302-7.

Caldas, H., Honsey, L.E. and Altura, R.A. (2005a). "Survivin 2alpha: A Novel Survivin Splice Variant Expressed in Human Malignancies." Mol Cancer 4(1): 11.

Caldas, H., Jiang, Y., Holloway, M.P., Fangusaro, J., Mahotka, C., Conway, E.M. and Altura, R.A. (2005b). "Survivin Splice Variants Regulate the Balance between Proliferation and Cell Death." Oncogene 24(12): 1994-2007.

Chantalat, L., Skoufias, D.A., Kleman, J.P., Jung, B., Dideberg, O. and Margolis, R.L. (2000). "Crystal Structure of Human Survivin Reveals a Bow Tie-Shaped Dimer with Two Unusual Alpha-Helical Extensions." Mol Cell 6(1): 183-9.

Chen, Z., Naito, M., Hori, S., Mashima, T., Yamori, T. and Tsuruo, T. (1999). "A Human Iap-Family Gene, Apollon, Expressed in Human Brain Cancer Cells." Biochem Biophys Res Commun 264(3): 847-54.

Clarke, P.R. (2005). "The Crm De La Creme of Mitosis." Nat Cell Biol 7(6): 551-2.

Clem, R.J., Fechheimer, M. and Miller, L.K. (1991). "Prevention of Apoptosis by a Baculovirus Gene During Infection of Insect Cells." Science 254(5036): 1388-90.

Clem, R.J. and Miller, L.K. (1994). "Control of Programmed Cell Death by the Baculovirus Genes P35 and Iap." Mol Cell Biol 14(8): 5212-22.

Conway, E.M., Pollefeyt, S., Cornelissen, J., DeBaere, I., Steiner-Mosonyi, M., Ong, K., Baens, M., Collen, D. and Schuh, A.C. (2000). "Three Differentially Expressed Survivin Cdna Variants Encode Proteins with Distinct Antiapoptotic Functions." Blood 95(4): 1435-42.

Dasso, M. (2001). "Running on Ran: Nuclear Transport and the Mitotic Spindle." Cell 104(3): 321-4.

Dasso, M. (2002). "The Ran Gtpase: Theme and Variations." Curr Biol 12(14): R502-8.

Deveraux, Q.L. and Reed, J.C. (1999). "Iap Family Proteins--Suppressors of Apoptosis." Genes Dev 13(3): 239-52.

Deveraux, Q.L., Roy, N., Stennicke, H.R., Van Arsdale, T., Zhou, Q., Srinivasula, S.M., Alnemri, E.S., Salvesen, G.S. and Reed, J.C. (1998). "Iaps Block Apoptotic Events Induced by Caspase-8 and Cytochrome C by Direct Inhibition of Distinct Caspases." Embo J 17(8): 2215-23.

Deveraux, Q.L., Takahashi, R., Salvesen, G.S. and Reed, J.C. (1997). "X-Linked Iap Is a Direct Inhibitor of Cell-Death Proteases." Nature 388(6639): 300-4.

Engels, K., Knauer, S., Metzler, D., Simf, C., Struschka, O., Bier, C., Mann, W., Kovacs, A. and Stauber, R. (2007). "Dynamic Intracellular Survivin in Oral Squamous Cell Carcinoma: Underlying Molecular Mechanism and Potential as an Early Prognostic Marker." J Pathol 211(5): 532-40.

Englmeier, L., Fornerod, M., Bischoff, F.R., Petosa, C., Mattaj, I.W. and Kutay, U. (2001). "Ranbp3 Influences Interactions between Crm1 and Its Nuclear Protein Export Substrates." EMBO Rep 2(10): 926-32.

Fahrenkrog, B., Koser, J. and Aebi, U. (2004). "The Nuclear Pore Complex: A Jack of All Trades?" Trends Biochem Sci 29(4): 175-82.

Fangusaro, J.R., Jiang, Y., Holloway, M.P., Caldas, H., Singh, V., Boue, D.R., Hayes, J. and Altura, R.A. (2005). "Survivin, Survivin-2b, and Survivin-Deitaex3 Expression in Medulloblastoma: Biologic Markers of Tumour Morphology and Clinical Outcome." Br J Cancer 92(2): 359-65.

Ferlay, J., Autier, P., Boniol, M., Heanue, M., Colombet, M. and Boyle, P. (2007). "Estimates of the Cancer Incidence and Mortality in Europe in 2006." Ann Oncol 18(3): 581-92.

Ferrando-May, E. (2005). "Nucleocytoplasmic Transport in Apoptosis." Cell Death Differ 12(10): 1263-76.

7 Appendix

7.1 List of figures and tables