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**THE ROLE OF BIDIRECTIONAL
NUCLEOCYTOPLASMIC TRANSPORT IN
APOPTOSIS**

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LIST OF ABBREVIATIONS

bp	base pair
°C	degree Celcius
g	g-force
hr	hour
kDa	kilodalton
mg	milligram
min	minute
μm	micrometer
mm	millimeter
mM	millimolar
nM	nanomolar
rpm	revolutions per minute
%	percent
μg	microgram
μl	microliter
AIF	Apoptosis-inducing factor
Apaf-1	Apoptosis protease activating factor-1
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BIR	Baculovirus IAP repeat
BRCA1	Breast cancer type 1 susceptibility protein
BSA	Bovine serum albumin
CAD	Caspase-activated DNase
CARD	Caspase activation and recruitment domain
Cdk1	Cyclin dependent kinase 1
c-Ab1	Abelson murine leukemia viral onco- gene homolog 1
cDNA	Complementary deoxyribonucleic acid

CENP-E	Centromeric protein E
CFP	Cyan fluorescence protein
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHX	Cyclohexamide
CPC	Chromosome Passenger Complex
CRM1	Chromosomal region maintenance 1
DAPI	4',6-diamidino-2-phenylindole, nuclei marker
DAXX	Death-domain-associated protein
DEDD	Death effector domain-containing protein
DFF40	DNA fragmentation factor, 40 kDa
DFF45	DNA fragmentation factor, 45 kDa
DIO-1	Deiodinase iodothyronine, type I
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DYRK2	Dual-specificity tyrosine- phosphorylation-regulated kinase 2
EDTA	Ethylenediamine tetraacetic acid
EGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol tetraacetic acid
FADD	Fas-associated death domain
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FG	Phenylalanine-glycine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCN5	K(lysine) acetyltransferase 2A (also known as KAT2A)
GDP	Guanosine 5' - diphosphate
GFP	Green fluorescent protein
GTP	Guanosine 5' - triphosphate
HAT	Histone acetyltransferase
HDAC4	Histone deacetylase 4

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse radish peroxidase
HBXIP	Hepatitis B virus x protein-interacting protein
IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of caspase activated DNase
IgG	Immunoglobulin G
JNK	Jun amino-terminal kinases
KCl	Potassium chloride
MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukemia sequence 1
MgCl ₂	Magnesium chloride
Mst-1	Mammalian sterile 20-like 1 kinase
NaCl	Sodium chloride
NES	Nuclear export signal
NF-κB	Nuclear factor-kappa B
NLS	Nuclear localization signal
NPC	Nuclear pore complex
NTR	Nuclear transport receptor
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PAK2	p21 activated kinase 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PF	Paraformaldehyde
PI-9	Proteinase Inhibitor 9
PKC	protein kinase C
PS	Penicillin/Streptomycin
PTEN	Phosphatase and tensin homolog
RanBP	Ran-binding protein
RanGAP	Ran-GTPase activating protein
RanGEF	Ran-Guanine exchange factor

RING	Really interesting new gene
Rb	Retinoblastoma
RCC1	Regulator of chromosome condensation 1
RT	Room temperature
SAC	Spindle assembly checkpoint
SDS	Sodium dodecyl sulphate
TBST	Tris buffered saline with tween-20
TNF	Tumor necrosis factor
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain
Ts-IAP	Testis-specific inhibitor of apoptosis protein
UBA	Ubiquitin associated domain
UBC	Ubiquitin conjugation domain
UPS	Ubiquitin-Proteasome System
WT	Wild type
XAF1	XIAP-associated factor 1
XIAP	X-linked Inhibitor of apoptosis protein
YFP	Yellow fluorescence protein

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ABSTRACT

Apoptosis or programmed cell death is a tightly controlled process regulated by many signaling pathways; however, the mechanisms and cellular events that decide whether a cell lives or dies remain poorly understood. Here I showed that when a cell is under apoptotic stress, the pro-survival protein Survivin redistributes from the cytoplasm to the nucleus, thus acting as a physiological switch to commit the cell to apoptosis. The nuclear relocalization of Survivin is a result of inefficient assembly of functional RanGTP–CRM1–Survivin export complex due to apoptotic RanGTP gradient collapse. Subsequently, Survivin undergoes ubiquitination, which not only physically prevents its diffusion back to the cytoplasm but also facilitates its degradation. Together, this spatial and functional regulation of Survivin abolishes its cytoprotective effect toward the apoptotic executors and thus commits a cell to apoptosis. My data indicate that the withdrawal of Survivin is a novel and active physiological regulatory mechanism that tilts the survival balance and promotes the progression of apoptosis.

THESIS SUMMARY

This thesis is divided into four chapters. Chapter one is an introductory section that presents the general background of this study. First and foremost, the current understanding of programmed cell death or apoptosis is overviewed. This is followed by more detailed description of the molecular players, biochemical related and/or purported systems heavily involved in the execution of apoptosis. The chapter concludes with the aims of the study.

Material and Methods forms Chapter two. This section provides comprehensive details of all experimental procedures conducted in the study.

Chapter three presents an elaborate report of the experimental results obtained from the study. In this section, it was first shown that Survivin is crucially depleted from the onset of apoptosis. The chapter then focuses on the mechanistic aspects and the importance of the subsequent molecular events that followed culminating in the initiation and propagation of apoptosis.

Chapter four opens with an analysis of the goals and the results obtained from the study. The emerging role of the nucleocytoplasmic trafficking system as regulators of cell death, the potential of Survivin and issues concerning Survivin-based therapy is further discussed in this chapter.

CHAPTER 1 : INTRODUCTION

1.1 Apoptosis

Notwithstanding a host of varied morphological, biochemical and genetic characteristics depicting distinctive forms of cell death, apoptosis solely represents a systematic, efficient and methodical program of cellular suicide. Apoptosis or programmed cell death, albeit its ominous label, is a common and essential feature to all multicellular organisms to regulate cell numbers. As such, it plays fundamental roles in several key biological processes, including embryonic development, maintaining tissue and organ homeostasis throughout the adult life, immune responses and disease states upon dysregulation [1-4]. Distinguishing hallmarks of apoptosizing cells include chromatin condensation (pyknosis), nuclear DNA fragmentation (karyorrhexis), cell shrinkage and membrane blebbing.

The critical activation of the family members of cysteine aspartate proteases (Caspases) arbitrates and propagates the apoptotic process. Caspases are bisected into the upstream initiation caspases and the downstream effector caspases. Upon activation, these cell death executioners will direct the cleavage of their cellular substrates such as DNA repair enzymes, lamins, actin regulatory proteins, and Focal adhesion kinase (FAK), thus driving apoptotic cells towards irreversible cellular demise [5].

To date, two main pathways have been extensively mapped out by which mammalian cells undergoes apoptosis, namely the extrinsic pathway or the death receptor pathway and the intrinsic pathway or the mitochondrial pathway. The extrinsic pathway is activated upon the binding of ligands to the transmembrane death receptors at the cell surface such as tumor necrosis factor (TNF) and Fas receptor. Subsequently, the aggregation and clustering of these receptors recruit adaptor proteins like Fas-associated death domain (FADD) and pro-caspases -8 or -10, forming the multiprotein death-inducing signaling complex (DISC) and culminates with the activation of the aforementioned caspases which are later released into the cytoplasm to activate effector caspases [6]. On the other hand, the intrinsic pathway is initiated in response to a wide range of genotoxic, metabolic and other stimuli that lead to the increase in permeability and the loss of

mitochondrial integrity. Together with Apaf-1 (Apoptosis protease activating factor-1), the mitochondrial-released cytochrome *c* congregates in a large supramolecular heptameric complex called the apoptosome. The apoptosome recruits pro-caspase-9 and promotes its activation via allosteric change and dimerization, hence enabling the initiator caspase to cleave and further activate downstream effector caspases-3 and -7 [7-9].

As constituents of the tightly-controlled process, cellular factors comprised both of apoptosis stimulants and apoptosis inhibitors are crucially involved in regulation of apoptosis. The pro-apoptotic proteins include Bax, Apaf-1, p53, retinoblastoma (RB), and p21, whereas the anti-apoptotic factors are comprised of proteins like the Bcl-2 (B-cell lymphoma 2), Inhibitor of Apoptosis proteins (IAPs), Mcl-1 (Myeloid cell leukemia sequence 1), and NF- κ B. The interplay between these opposing factors controls cell fate decisions on whether or not a particular cell lives or dies.

1.2 Survivin

The family of evolutionary conserved pro-survival proteins, IAPs, is essential in protecting cells from entering apoptosis by negative regulation of downstream caspases [10-14]. Generally, all true IAPs are characterized by the presence of one to three copies of the Baculovirus IAP repeat (BIR) domains. Eight members of the IAPs had thus far been identified in humans, namely the XIAP (X-linked inhibitor of apoptosis), cIAP1, cIAP2, NAIP, Livin, Bruce/Apollon, Ts-IAP and Survivin (Fig. 1.1) [15, 16].

Survivin, with a predicted mass of 16.5 kDa, is the smallest member of the IAP family, containing a single ~70 amino acid BIR motif. It is encoded by 4 exons located along the human chromosome 17 at position q25. The tentative full-length pre-mRNA could further be alternatively spliced to generate 5 distinctive mRNAs and thus 5 isoforms of the Survivin proteins: Survivin (142 amino acids), Survivin 2 α (74 amino acids) Survivin 2 β (165 amino acids), Survivin 3 β (120 amino acids) and Survivin- Δ Ex3 (137 amino acids) [17-19]. The expression patterns of these Survivin isoforms vary with specific tissue origins. However, it is important to note

that the wild type Survivin transcripts predominates over the other isoforms in the cases of commonly used human cell lines such as HeLa, MCF7 and others [19, 20].

It is fascinating to note that while Survivin is a vital suppressor of apoptosis [14, 21, 22], it is also an integral component of the Chromosomal Passenger Complex (CPC), which directs mitotic activities [14, 23, 24]. The expression of Survivin is cell cycle-regulated and escalates to a maximum during the G2/M phase, consistent with its role in mitosis. Although Survivin is virtually undetectable in terminally differentiated adult tissues and dormant G₀ cells, it is prevalent in developing fetal tissues and actively dividing cells. Interestingly, it is also frequently over-expressed and/or deregulated in most neoplastic tissues [25-29]. As a result, Survivin is widely recognized as a leading research candidate in the development of prognostical tool and a prime target in cancer therapy [21, 30].

Given its low molecular weight, Survivin readily enters the nucleus by passive diffusion. Under normal circumstances, exclusion of Survivin from the nucleus depends on the interaction of the NES-containing Survivin, its transport receptor CRM1, and also the RanGTP/GDP axis. Hence, Survivin that diffuses into the nucleus are actively and rapidly exported back into the cytoplasm [31-33]. On a different note, endogenous Survivin turnover has been associated to the ubiquitination-mediated proteolysis by 26S proteasome [34, 35].

Figure 1.1 Diagram of BIR-domain containing proteins. (*Left*) Elaborate representation of Survivin-protein interactions and functional motifs. (*Right*) IAP family members. UBA: Ubiquitin-Association Domain, LRR: Leucine rich repeat domain, NOD: Nucleotide binding and oligomerization domain, UBC: Ubiquitin Conjugating domain, CC: Coiled coil domain, CARD: Caspase recruitment domain, RING: Really interesting new gene domain.

1.2.1 Survivin as the cell death regulator

Faithful as a member of the IAP, Survivin exhibits anti-apoptotic characteristics. From the molecular perspective, BIR domains generally facilitate protein recognition and arbitrate protein-protein recognition [36] and in particular modulating apoptotic factors such as the caspases. While the direct binding of Survivin to target downstream effector caspases is still a subject of vitriolic debate, a prevailing model ascertains the anti-apoptotic effect of Survivin by *in vivo* co-operative interactions with other cellular factors. Under the cytoprotective scheme of IAP-IAP complex, Survivin has been shown to interact, stabilize and synergistically cause the significant enhancement of XIAP activity to directly inhibit executioner caspases such as caspase-3 [37, 38]. Likewise, Survivin engages and asserts its inhibitory effect on the initiator caspase-9 indirectly utilizing Hepatitis B virus χ protein-interacting protein (HBXIP) as cofactor [10]. Another mechanism of action depicting the pro-survival role of Survivin involves the binding with Smac/DIABLO, which serves to sequester these natural IAP inhibitors from targeting XIAP and/or prevent their release from mitochondria [39, 40].

The aforementioned molecular pathways illustrating how Survivin exhibits its cytoprotectivity are by no means exhaustive. Clearly there is still much to discover regarding this enigmatic nodal protein as several lines of concrete evidences collectively point to the prominent role of Survivin in inhibiting apoptosis. It is important to note that cell death incited either through the intrinsic pathway or the extrinsic pathway are substantially suppressed by the artificial expression of recombinant Survivin in multiple cell culture systems [13, 19, 41-46]. In addition, *in vivo* animal studies revealed that transgenic expression of Survivin contributed to the protection against UV-irradiation-induced apoptosis [47]. Apart from expression related analysis, studies involving the physical or functional antagonization of Survivin by various molecular entities demonstrated spontaneous and/or sensitization to caspase-dependent apoptosis *in vitro* and also in animal tumour models [48-52]. Furthermore, elevated level of endogenous

Survivin has been reported to confer radiation resistance whereas depletion of Survivin sensitizes and renders cells more susceptible to irradiation-induced cell death [53, 54].

In line, the cytoprotectivity of Survivin has been proposed by various studies to be dependent on its subcellular localization [28, 35]. Moreover, clinical studies have orchestrated the correlation between nuclear retention of Survivin in transformed cells and positive prognosis for patients [27, 29, 55]. Survivin negatively modulates downstream caspases in the cytoplasm thereby preventing cells from undergoing apoptosis. Consistently, mutation in the NES region and forcible relocation of Survivin into the nucleus inhibits its role to prevent apoptosis [33, 35, 56, 57]. In sum, proper localization of Survivin is necessary for the protection of cells from apoptosis and/or to prevent the sensitization of cells towards stress-inducing or apoptotic stimuli.

1.2.2 Survivin as the mitotic facilitator

Mitosis marks the most remarkable and elaborate event in the life of actively proliferating cells to ensure that every pair of daughter cells produced from each divisions are genetically identical. In a little more than an hour, mitosis orchestrates major changes in multiple cellular components, signaling pathways being intricately activated and silenced on cues, and the timely prompting of the protein degradation processes leading to gross dynamic reorganization of the cell structure. The sequential occurrence of the 5 distinctive phases of mitosis begins with prophase, followed by prometaphase, metaphase, anaphase and eventually ends of with telophase assuming that the mitotic checkpoint does not detect any aberrance (Fig. 1.2). Prophase is characterized by chromosome condensation and the locomotion of the duplicated centrosomes towards opposite poles of the cell. Next, the nuclear envelope breaks down during prometaphase thereby granting access to the astral microtubules emanating from the centrosomes to centromeric region of the chromosomes. This search and capture process will continue until all the chromosomes are connected and aligned at the equatorial plane of the cell, commonly known as the metaphase plate. Anaphase is then triggered when proper

bipolar spindle attachment to the kinetochores are achieved at the end of metaphase. In an anaphasic cell, sister chromatids separate and move towards opposite poles of the cell. Finally, nuclear envelope reforms around the segregated daughter chromosomes during telophase. This process is followed by chromatin decondensation and cytokinesis, hence giving rise to two identical daughter cells.

Although Survivin was first recognized as an IAP, its expression, synthesis and turnover are tightly regulated in a cell-cycle dependent manner, thus hinting possible roles of Survivin in the regulation of mitosis [14, 58-60]. Transcription of Survivin commences in G1, peaks at G2/M, and breaks down thereafter. Additionally, the intriguing subcellular localization of Survivin bears uncanny resemblance to that of the CPC, further lending support to the notion of Survivin as a mitotic facilitator [24, 61-63]. In accordance to the function of CPC, Survivin is localized at the inner centromeres during prophase and metaphase, shifted to the developing midzone microtubules during anaphase and finally deposited onto the midbody during telophase and cytokinesis [14, 22] (Fig. 1.2). Once mitosis is over and the nuclear envelope reforms, Survivin is predominantly cytoplasmic throughout interphase [31-33].

A critical requirement for Survivin's mitotic function was later identified to be the phosphorylation on threonine residue (Thr34). O'Connor et al [64] had demonstrated that Survivin coimmunoprecipitates with cyclin-dependent kinase-1 (CDK1) and is phosphorylated by it at a single threonine residue Thr34 *in vitro*. Under normal circumstances, the expression of Survivin is the highest at G2/ M phase coinciding with the window period where CDK1 is active. Hence, with rapid phosphorylation of Survivin at Thr34 by CDK1, Survivin interacts with Borealin, INCENP, and Aurora-B kinase to form the functional CPC, which later targets itself to the kinetochores [21, 23, 31, 65]. Consistent with the roles of CPC, studies involving the downregulation/ablation/knockout/functional attenuation of Survivin revealed severe defects in mitotic spindles assembly and maintenance, compromised spindle checkpoints surveillance, aberrant chromosome movements, increase in ploidy, and the inability to complete cytokinesis [22, 66-70]. Moreover, embryonic lethality observed in Survivin-knockout mice further emphasizes its

crucial role in cell division since mitosis is the key indispensable feature of development [24].

Figure 1.2 Localization of Survivin in cell division. Hela cells expressing Survivin-myc were fixed at different stages of mitosis. Myc was stained red, microtubules green and the DNA was counterstained with DAPI (blue). Survivin initially localizes at the inner centromeres from prophase to metaphase, and then relocates to the midzone microtubules between the separating chromosomes during the transition from metaphase to anaphase. During telophase and cytokinesis, Survivin is conjugated at the midbody and ultimately discarded when the daughter cells separate. After nuclear envelope reformation at the end of mitosis, Survivin is predominantly found in the cytoplasm throughout interphase. Scale bar denotes 10µm.

1.3 Survivin and Cancer

Cancer is the dreaded term used for a complex group of genetic diseases in which abnormal cells divide out of control and have the capability to metastasize to other tissues. There are over one hundred types of cancer, each behaving differently depending on the cell type from which they originate. The difference between cancerous cells and normal cells is that instead of dying when they should, these transformed cells continue to grow and divide uncontrollably leading to the incessant formation of new abnormal cells. The American Cancer Society made a staggering statistical claim that half of all men and one-third of all women in the United States of America will develop cancer during their lifetimes. It is undeniable that cancer poses a serious global health problem culminating in significant portion of mortality rate worldwide.

Survivin strikes as a clear cancer-specific gene considering the fact that it is overexpressed in practically every human tumour examined. In contrast, it is untraceable in most adult tissues except at low levels in the thymus, CD34+ bone marrow-derived stem cells, basal colonic epithelium, and placenta [41]. The conspicuous overexpression of Survivin was reported in human tumours originated from the digestive system: esophagus, stomach, colon, pancreas and liver [71-75]; reproductive system: ovary and uterus [76, 77]; nervous system: neuroblastoma [43, 78, 79]; integumentary system: breast, soft tissues sarcomas, melanoma, and non melanoma [50, 80-84]; respiratory system: lung [27, 29, 55, 85]; and circulatory/lymphatic system: large cell non-Hodgkin's lymphoma, and leukemias [86-89].

Clinical retrospective analysis of cancer patients with positive Survivin overexpression implicated reduced overall survival [71-73, 77, 82-86, 90], elevated rates of relapse incidences [91], poor prognostical disease progression [43, 78, 87, 89, 92], and increased chemoresistance [72, 93]. Interestingly, the pro-survival effect of Survivin in the cancerous cells is also associated to its subcellular localization in the cytoplasm for studies involving human cancer cell lines [28, 35] and patient-based research alike [27, 29, 55]. Recently, it was also reported that

high cytoplasmic Survivin expression in parotid gland cancer correlated to a poor 5-year disease free and increased mortality rate [94]. Similarly, in the case of head and neck squamous cell carcinomas, the prevalence of cytoplasmic Survivin are linked to poor prognosis [95]. On a different note, Survivin had also been shown to play a pivotal part in the angiogenesis development [96-98].

Hence, it is not surprising that Survivin is an attractive target for potential therapeutic manipulations to combat cancer, with large pharmaceutical companies investing heavily to generate impactful Survivin antagonist (some of which are already in various phases of clinical trials). Couple the pro-survival role of Survivin to its specific expression in neoplastic tissues, and to top it off with its critical angiogenic functionalities, Survivin presents itself as the Holy Grail of cancer therapies.

1.4 Ubiquitin-proteasome proteolysis

Approximately 80% of intracellular proteins particularly the damaged, misfolded or frequently turned-over proteins are degraded through the supramolecular machinery, Ubiquitin-Proteasome System (UPS). The 20S core particle (base) and the 19S regulatory particle (lid) constituted the highly organized ~2,500 kDa 26S proteasome [99]. The ubiquitin, on the other hand, is a small protein of only 8 kDa. Key biological processes such as transcriptional regulation, cell-cycle progression, and signal transduction regulated by this highly conserved system in all eukaryotes. As such, aberrations in the UPS would result in pathological conditions like malignant transformation and neurodegenerative diseases [100].

For a protein destined for proteolysis, it must be covalently conjugated with ubiquitin molecules via the isopeptide bond between the C-terminal glycine of ubiquitin and an accessible lysine residue of the substrate. The whole process of ubiquitin-tagging entails sequential actions of three enzymes, namely the E1 (ubiquitin activating enzyme) which activates the glycine-76 of the ubiquitin, the E2 (ubiquitin conjugating enzyme) that temporarily attaches to the activated ubiquitin, and the E3 (ubiquitin ligase) that recruits E2 and the substrate together

thus enabling the transfer of the activated ubiquitin to the substrate [101, 102]. Polyubiquitin chains are then generated upon successive rounds of ubiquitination. These polyubiquitin chains can be further subdivided into K48-polyubiquitin chains (ubiquitins that are linked through lysine-48 for degradation purpose) and K63-polyubiquitin chains (lysine-63 conjugated ubiquitins for protein kinase activation, signaling purposes, DNA repair and vesicle trafficking) [101, 102].

As far as proteasomes are concerned, although these polypeptide-disintegrating units are present throughout the cell, there are distinctive differences in terms of relative abundance across different cellular compartments. With regards to this study, it is important to note that there is a pattern of proteasome distribution across the nucleocytoplasmic boundaries during the cell cycle [103]. Proteasomes start to accumulate in the nucleus of the cell during S phase and the nuclear abundance peaks during the G2 phase. Upon entry to mitosis, the nuclear envelope breaks down resulting in homogenous scattering of the proteasomes. Hence, once the nuclear membrane reforms, proteasomes remains predominantly cytoplasmic during G1. Following that, it was also reported that all the necessary components of UPS for it to be functionally active for protein degradation are found residing in the cell nucleus [104, 105]. In support, the cell cycle dependent build-up of proteasomes in the nucleus is observed in HeLa and PtK2 cells [106]. Consistent with the importance of evolutionary conserved proteins, proteasomes are found predominantly nuclear especially during the later phases of interphasic nucleus of *Schizosaccharomyces pombe* and ascidian *Halocynthia roretzi* [107, 108]. Nevertheless, the distribution of proteasomes is part of the modulatory systems in place together with the availability of substrate specific E2 and E3 proteins (both regulated in time and space) to ensure proper control of protein degradation.

1.5 Regulation of apoptosis by ubiquitination

It is becoming increasingly clear that the UPS is involved in critical mechanistic pathways in regulating apoptosis by modifying and/or degrading key regulatory proteins along the apoptotic-survival axis [109-112]. Early evidences linking the UPS to cell death arose from the study of developmentally programmed atrophy and intersegmental muscles degeneration in hawkmoth where the expression of ubiquitin was found to be significantly elevated just before distinct apoptotic morphological changes appeared [113, 114]. Concurring data from other model systems emerged later on implicating the increase in the quantity, activation status, and function of UPS-associated proteins during the propagation of cell death [115, 116]. Additionally, apoptosis is effectively blocked in lymphocytes by proteasomal inhibitors when challenged with DNA damage agents and glucocorticoid [116, 117], further lending support to the notion that ubiquitination is a major active component of the cell death regulatory mechanism.

In terms of UPS-modulated cell cycle regulation and response to DNA damage, the potent tumor suppressor p53 is responsible to induce cell cycle arrest, senescence and apoptosis upon stabilization [118-120]. Nonetheless, a more defining example of UPS-regulated apoptotic pathway is the discovery of RING domains located within proteins from the IAP family. XIAP, cIAP1, cIAP2, Livin and Ts-IAP all possess the RING domain, thus enabling them to function as E3 ligases to facilitate the ubiquitination and the subsequent degradation of interacting proteins such as caspases and Smac/DIABLO [121]. While this is true from the cytoprotection role of the IAPs, it is intriguing to note that the RING domains also mediate autoubiquitination of XIAP, cIAP1 and cIAP2 upon apoptotic stimulation. These aforementioned IAPs are degraded in thymocytes undergoing glucocorticoid/etoposide-induced apoptosis, of which could be effectively rescued by the addition of proteasome inhibitors and hence promotes survival [122]. Considering the opposing disparity of RING-mediated outcomes of the IAPs, clearly some form of apoptotic threshold exist to dictate the fate of the IAPs, whether to continue protect the cell and promote survival or to withdraw and propagate the death progression. Nevertheless, it is still incompletely understood

on what would then be the fate and role of IAPs without the RING domain such as Survivin, NAIP or BRUCE, and other non-IAP interacting pro-survival proteins upon apoptosis induction.

To a smaller extent, depletion of other anti-apoptotic factors besides the IAPs via the ubiquitin-proteasomal pathway may also assist in the progression of apoptosis. The anti-apoptotic factor Mcl-1, which is a member of the Bcl-2 family, was reportedly degraded via the UPS to promote the relocation of Bax to the mitochondria eventually leading to *cytochrome c* release and subsequent caspase activation [123, 124]. Apart from that, the pro-survival proteins Ku70 and Ku80 were demonstrated to be a target for proteasomal degradation during the onset of Staurosporine-induced apoptosis in various cell types [125]. Taken together, it is apparent that the UPS constitutes a major mechanistic pathway in the regulation of apoptosis.

1.6 Nucleocytoplasmic transport

Apart from the presence of a nucleus, what differentiates a eukaryote from a prokaryote is the existence of multiple separate membrane-encapsulated compartments that enables specific cellular functions to take place within them. Despite the newly acquired sophistication and advantages, a new challenge presents itself in the form of remote communication between each organelles and the rest of the cell. To circumvent this problem, eukaryotes express a host of integral transmembrane-translocators, channels and other forms of transporters and delivery molecules, each anchored to the membranes of these highly organized compartments. These markedly specialized transport-associated molecules serve to facilitate efficient trafficking and yet at the same time do so without compromising the integrity of the organelles.

Naturally the heaviest traffic of all transpires across the boundary between the interphasic nucleus and the cytoplasm. The presence of nuclear envelope in eukaryotic cells serves as a physical separation in compartmentalizing the nuclear genomic materials from the cytoplasmic substances. The biophysical nature of this semi-permeable barrier necessitates a competent and controlled nucleocytoplasmic

trafficking of proteins and RNAs through the nuclear pore complex (NPC) for basic biological processes to occur. The core nucleocytoplasmic transport machinery generally consists of cargoes, nuclear transport receptors (NTRs) and also the key regulatory protein Ran [126].

1.6.1 Nuclear pore complex (NPC)

The site of bidirectional exchange between the nuclear and cytoplasmic macromolecules is positioned at the NPC, which spans across the inner and outer nuclear membrane. The NPC is a highly organized and massive proteinaceous structure consisting of more than 100 different polypeptides [127]. Geometrically, it can be subdivided into three portions; the octagonally symmetric cylindrical cytoplasmic filaments, the middle planar pseudo-symmetrical region across the nuclear pore, and the nuclear basket scaffold that extends into the nucleoplasm [128]. It is through these complexes that the regulated traffic of information occur and each NPC has been estimated to be able to manage the transport macromolecules in the hundreds every single minute in both directions [129].

Nucleoporins (Nups) comprising the biggest group of family of proteins lining the NPC is important in maintaining the passageway especially for proteins and RNAs whose size is above the cut-off limit for passive diffusion to take place. Basically, two forms of transport occur through the NPCs, namely the passive transport (for molecules ≤ 40 kDa in size) which is required to continuously shuttle water, essential metabolites and ions between the 2 compartments, and also the active signal-mediated transport for substantially large cargoes above 40 kDa.

A central theme to the NPC's selective permeability stems from the ordered positions of hydrophobic patches (FG, GLFG or FxFG motifs) as contributed by the various nucleoporins in the complex. These Nups are tethered on one side of the NPC such that their FG-repeat containing domains juts out into the central pore cavity, nucleoplasm and also the cytoplasm [130]. One particular model proposes that the selectivity is achieved by self-interacting FG (phenylalanine-glycine)-repeats themselves creating a mesh-like filter perforated with approximately 10 nm-wide openings in the central pore channel, thus enabling only small molecules

to cross over passively but excluding other larger inert compounds. Nonetheless, in the case of signal-mediated active transport, NTR-cargo complex traverses the meshwork by replacing the FG-FG contacts of the Nups with hydrophobic patches present on the NTR itself. As a result, the NTR-cargo complex literally “dissolves” through the mesh and through the NPC. The meshwork reforms as the NTR translocates deeper into the channel allowing the FG-repeats to resume its contacts [131, 132]. Alternatively, it has also been suggested that non-linked FG-repeats found protruding towards the cytoplasm and the nucleoplasm sift out macromolecules via thermal motion. NTR-coupled cargoes are able to pass through this barrier by FG interactions, hence they are more likely to enter into the nucleus [133].

1.6.2 The roles of nuclear transport receptors (NTRs)

The karyopherin- β family is a group of evolutionary conserved transport receptors that facilitates the import (importins) and export (exportins) of most proteins, ribosomal subunits and some RNAs across the nuclear envelope [134]. Majority of karyopherins such as importin- β are able to directly interact with their cargoes. Nonetheless, in some cases, adaptor proteins may also be used for the assembly of karyopherin-cargo complex [135]. The assembled karyopherin-cargo complex traverses through the NPC by forming multiple, random, low-affinity interactions with the FG-repeat containing nucleoporins that lines the nuclear pore channel.

Briefly, for the import of protein into the nucleus, importin first recognizes and binds to the basic Nuclear Localization Signal (NLS) present on the cargo itself. The importin-cargo complex will then translocate into the nucleus via the NPC. At the nucleoplasmic side, when RanGTP is encountered, importin preferentially binds with high affinity to RanGTP to form the importin-RanGTP complex instead. As a result, the cargo dissociates from the complex and is released within the nucleus. The importin will be subsequently recycled back to the cytoplasm to mediate the next round of transport (Fig. 1.3A) [136].

Conversely, export cargoes contain leucine rich-Nuclear Export Signals (NES) which are recognized by the exportins. Binding of exportin to the cargo occurs co-operatively with RanGTP to form the RanGTP-exportin-cargo complex. This mode of export complex assembly is necessary as transport receptors by themselves have low affinity towards –NES harbouring cargoes or Ran [137]. A crystallized fragment of CRM1 (exportin-1) revealed a flexible loop in the centre of the protein, which masks the cargo-binding site and obstructs the CRM1 and Ran interaction. It was proposed that interaction of CRM1 with Ran changes the confirmation of the loop such that stable trimeric complex formation is favoured [138]. The assembled RanGTP-exportin-cargo complex translocates out of the nucleus where it will encounter RanGAP and RanBP1. Hydrolysis of RanGTP to RanGDP mediated by RanGAP and RanBP1 will promote the dissociation of the trimeric complex at the cytoplasmic side. This will release the cargo into the cytoplasm and the exportin will be transported back into the nucleus (Fig. 1.3B) [136].

1.6.3 RanGTP gradient

The maintenance and the directionality of the nucleocytoplasmic transport are crucially governed by the distribution of small GTPase Ran. Although Ran has important roles in mitotic spindle assembly and nuclear envelope assembly, Ran is critically involved in nuclear transport by regulating the assembly state of karyopherin-cargo complexes depending on its bound guanine-nucleotide state. Ran exists as either a GDP- or GTP-bound state just like many of members of the small GTPases family. As molecular switches pertaining to the nucleocytoplasmic transport, the import of molecules into the nucleus requires the binding of importins with RanGTP such that the cargo can be released within the nucleus. In addition, hydrolysis of RanGTP is necessary to dissociate the trimeric complex for the export of molecules out of the nucleus.

The guanine-bound state of Ran is modulated by Ran-binding proteins, which localize in distinctive subcellular compartments of the cell. RanGEF (Ran-Guanine-Exchange-Factor) the presence of RanGEF in the nucleus functions by

facilitating the formation of RanGTP. The generation of RanGTP *in vivo* is in part achieved through the coupling of RCC1-mediated nucleotide exchange on Ran with chromosome docking in the nucleus [139]. On the other hand, RanGAP (Ran-GTPase-Activating-Protein) together with RanBP1 (Ran-Binding-Protein-1) are present in the cytoplasm and they are required for catalyzing the hydrolysis of RanGTP to RanGDP. As such, the RanGTP gradient is further sharpened across the nuclear envelope in interphase. Taken together, a steep concentration gradient of RanGTP is established across the nucleocytoplasmic compartments [136, 140, 141]. It should also be noted that RanGDP is actively transported by nuclear transport factor 2 (NTF2) into the nucleus to be converted into RanGTP [142, 143]. Collectively, during interphase, a steep RanGTP gradient is necessary to regulate the directionality and serves as a track for effective nucleocytoplasmic trafficking.

Figure 1.3 Schematic illustration of nucleocytoplasmic trafficking. (A) Nuclear import. Importin recognize and binds to its NLS-containing cargo substrates in the cytoplasm. The resultant import-complex is then translocated into the nucleus. Inside the nucleus, the presence of abundant RanGTP favors the higher affinity

binding between Importin and RanGTP, thus releasing the cargo. (B) Nuclear export. Exportin will interact with RanGTP and cargo proteins containing leucine-rich nuclear export signal (NES). Formation of the trimetric export complex occurs co-operatively and the resultant complex is translocated to the cytoplasm. At the cytoplasmic side of the NPC, RanBP1 and RanGAP catalyses the hydrolysis of GTP on Ran, which then leads to the disassembly of the export complex, thus releasing the cargo into the cytoplasm.

1.7 Nucleocytoplasmic transport and apoptosis

The significance of apoptosis or programmed cell death is never undermined given its central role in normal development and homeostasis of multicellular organism. While apoptosis continues to be a field of active and extensive research, ambiguity regarding the mechanism behind the regulations of cell death by the nuclear transport machinery remains unresolved.

One of the key focuses in regards to nucleocytoplasmic transport and apoptosis lies in the deregulated trafficking of shuttling proteins as a result of compromised permeability of the nuclear envelope in the course of cell death. Prevailing evidences all point towards the caspase-mediated cleavage of specific nucleoporins both positioned peripherally or within the core of the NPC during apoptosis [144, 145]. However, it should be highlighted that the depletion of distinct nucleoporins, in particular Nup153 and hence the leaky nuclear membrane attribute was only observed during the later phases of apoptosis [146-148].

The availability and accessibility of main apoptotic players are also tightly regulated by nuclear transport, although the question on why and how most of these mechanistic pathways are achieved still remains incompletely elucidated. It has been reported that in dying cells proteins such as DEDD, TRADD, DIO-1, Apaf-1, Mst1, GAPDH, Apoptin, PAK2, Helicard, Caspase-3, Caspase-6, AIF, Granzyme B, Proteinase Inhibitor-9 (PI-9), PTEN, HDAC4, DFF40, FADD, and PEA-15 translocates from the cytoplasm to the nucleus. On the contrary, there is also the redistribution of proteins such as DAXX, Caspase-2, and BRCA1 that relocates from nucleus to the cytoplasm during apoptosis. While the list is by no means exhaustive, it serves as a clear indication that key regulatory proteins involved in apoptosis undergo dramatic reshuffling to coordinate the efficient and irreversible demise of the cell [149-152].

Another collection of proteins simply known as apoptotic kinases including protein kinase C (PKC) δ , Abelson murine leukemia viral onco- gene homolog 1 (c-Abl) and dual-specificity tyrosine- phosphorylation-regulated kinase 2 (DYRK2) has been shown to be transported into the nucleus from the cytoplasm upon genotoxic stress-induced apoptosis [153]. Consequently, once these activated pro-

apoptotic kinases enters into the nucleus, they proceed to promote the expression of a host of other apoptosis-related genes thereby pivotally influencing the progression of cell death.

From a new and substantially different standpoint, previously, we have discovered that during the initiation stages of apoptosis, there is indeed a regulatory pathway linking histone modifications to the shutting down of RanGTP gradient-mediated nucleocytoplasmic transport system [154]. In brief, I found that the decreasing nuclear RanGTP level correlates with the immobilization of RCC1 on the chromosomes during the early stages of apoptosis. Loss of function of RCC1, inferred by apoptotic histone H2B phosphorylation, eventually leads to the collapse of nucleocytoplasmic trafficking system. Consequently, failure to import NF- κ B into the nucleus prohibited its anti-apoptotic activity, which leads to eventual and inevitable cell death (Fig. 1.4).

Figure 1.4 Phosphorylation of H2BS14 leads to impairment of nuclear import. Mst1 is activated by caspases upon exposure to apoptotic stimuli. The activated Mst1 translocates to the nucleus and phosphorylates histone H2B at Ser 14, which leads to the impairment of RCC1 mobility. The immobilization of RCC1 traps it in a binary complex with Ran, which affects the turnover of free RCC1 to catalyse another round of nucleotide exchange. Thus RanGTP production is affected. The diminished level of nuclear RanGTP results in inactivation of the nuclear transport machinery. As a consequence, NLS containing proteins, including NF- κ B-p65, remain bound to importins α and β in the cytoplasm. *Adapted from [155].*

1.8 Aim of present study

As the balance between the positive pro-survival factors and the negative pro-apoptotic factors is crucial in determining the life and death of a cell under stress, I asked what might be the regulation that tips off this delicate balance. However, the fate and functionalities of existing pro-survival factors and proteins are incompletely elucidated during cell death. Considering the antagonistic nature of these factors, it is conceivable that some forms of modulatory mechanisms are in place to negate the effect of the anti-apoptotic proteins and to intensify the activation of pro-apoptotic proteins for the progression of programmed cell death. Hence, I hypothesized that active withdrawal and/or inhibition of the positive factors could be a prominent regulatory feature when cells are committed to apoptosis. The study herein describes a novel modulatory pathway mediating the removal of prevailing pro-survival protein Survivin during the onset of apoptosis by the impaired nuclear export processes that actively contributes towards the demise of the cell.

I discovered that there is an unexpected drastic decline of the anti-apoptotic Survivin protein level upon cell death induction. Additionally, endogenous Survivin was found to redistribute from the cytoplasm into the nucleus of apoptotic cells. Following that, I demonstrated that the accumulation of the NES-containing Survivin in the nucleus is associated with the collapse of RanGTP gradient during the early stages of apoptosis. To further elucidate the mechanism behind this observation, the RanGTP-Survivin-CRM1 pathway was examined. Hela cells transfected with Flag-Survivin immunoprecipitated significantly lower levels of CRM1 when induced to undergo apoptosis. This goes on to show that the decrease of RanGTP level in the nucleus critically impairs the co-operative binding capacity of the trimeric complex components leading to the shutting down of nuclear export pathway. In support, RNAi silencing of Mst1 not only restores the RanGTP gradient but also reinstates the proper cytoplasmic localization of Survivin even in the presence of apoptotic stimuli. Although it is known that normal Survivin-turnover occurs in the nucleus, we reasoned that the enforcement of Survivin into the nucleus during the onset of apoptosis effectively increases its molecular weight

by polyubiquitination and promotes its degradation by the ubiquitin-proteasome proteolytic pathway. Finally, I showed that compartmentalization of Survivin in the nucleus during apoptosis prevents it from interacting with the effector caspase-3 which are prevalent in the cytoplasm. In other words, the limited cytoprotectivity of mislocalized nuclear Survivin may yet be one of the factors that contribute actively towards the initiation and pivotally influence the propagation of apoptosis.

CHAPTER 2 : MATERIALS AND METHODS

2.1 Cell culture, transfection, and drug treatments

Hela cells and H1299 were obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (Gibco, Invitrogen, USA) containing 10% fetal bovine serum (BSA) (Hyclone, USA) and 1% Penicillin/Streptomycin (Gibco, Invitrogen, USA) at 37°C in a humidified atmosphere with 5% carbon dioxide.

Transfection procedures were done using Lipofectamine 2000 according to manufacturer's instruction. (Invitrogen, USA). The ratio of DNA (μg):Lipofectamine™ 2000 (μl) used in each transfection mixture was 1:3.

For VP16 treatment, 10 mg/ml etoposide (Sigma, USA) in dimethyl sulfoxide (DMSO) stock was diluted in medium to a final concentration of 20 $\mu\text{g}/\text{ml}$. MG132 and Cyclohexamide (both from Sigma, USA) were diluted with buffers to a final working concentration of 2 μM and 20 $\mu\text{g}/\text{ml}$ respectively. For Leptomycin B (Sigma, USA) treatment, the stock solution of 5 $\mu\text{g}/\text{ml}$ in 100% ethanol was diluted with buffers to a final working concentration of 5 ng/ml.

2.2 Immunofluorescence Microscopy

Cells seeded onto 22 mm coverslips were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min and permeabilized with 0.5% Triton X-100 (USB Corp, USA) in PBS for 5 min. After washing three times with PBS, cells were blocked in 4% BSA (Sigma, USA) for one hour at room temperature (RT) and then incubated with primary antibody diluted in TBST (50mM Tris, pH7.6, 150 mM NaCl, 0.1% Tween-20) plus 4% BSA. Following washes with TBST, the cells were incubated with appropriate secondary antibodies and incubated at RT for 1 hour in the dark. Cells were then mounted onto glass slides using ProLong gold antifade reagent containing DAPI (Invitrogen, USA).

Images were acquired and analyzed using an Axiovert 200M inverted microscope (Carl Zeiss, Germany) and Axiovision 4.6 software.

2.3 Fluorescence Resonance Energy Transfer (FRET)

Lab-Tek* Chambered cover glasses (Nunc) were used to seed the cells. FRET was then performed on a Zeiss LSM 510 Meta confocal microscope equipped with Incubator S (Pecon, Germany). During the experiment, the temperature and CO₂ level was maintained at 37°C and 5% respectively by placing the seeded cells in Incubator S on the microscope stage. Acceptor Photobleaching method was used for FRET analysis [156]. Five consecutive images were obtained at 1% of the laser intensity in CFP and YFP channels before and after YFP photobleaching by repeatedly scanning the designated area at 75% laser intensity. The background CFP fluctuations during FRET were determined from the unbleached control cells present in the same field. FRET efficiency was calculated as $E_F\% = (I_6 - I_5) \times 100 / I_6$ where I_5 and I_6 represent the CFP fluorescence intensity of the fifth and sixth images immediately before and after YFP photobleaching. At least 15 cells were analyzed for each experimental set. Results were presented as mean percentage FRET efficiencies $\pm$ standard deviation.

2.4 Preparation of cell extracts, immunoblotting and immunoprecipitation

For the collection of whole cell lysates, cells were washed with PBS and lysed by M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, USA) containing 10% Complete EDTA-free Protease Inhibitor Cocktail (Roche, Switzerland) and 0.1% phosphatase cocktail inhibitor 1 and 2 (Sigma, USA). Nuclear Cytoplasmic fractions were prepared using NE-Per Nuclear and Cytoplasmic Extraction (Thermo Scientific, USA) according to the manufacturer's protocol.

2x sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue) was added to whole cell lysates, nuclear lysates and cytoplasmic lysates before boiling the mixture for 10 min. Equal amounts of protein were resolved on SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Biorad laboratories, USA). Membranes were blocked with 10% skim milk in TBST for two hours at RT and probed with specific primary antibodies diluted in blocking solution at 4°C overnight.

Membranes were washed thrice with TBST before incubating with horseradish peroxidase-conjugated secondary IgG antibodies (Invitrogen, USA). After washing with TBST, immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Biosciences, USA).

For immunoprecipitation, Hela cells transfected with Flag-Survivin were incubated with or without VP16 for 24 hours. These cells were then lysed with M-PER and the cell lysates were incubated with anti-Flag antibody for 1 hour at room temperature. Rec-Protein-G-Sepharose 4B (Zymed, USA) were added to each experimental set. The resultant mixtures were further incubated for 1 hour at room temperature before repeated washing with Wash buffer (TBS with 100 mM NaCl) to rid unbound proteins. Immunoprecipitated proteins were separated by SDS-PAGE prior to western blotting analyses.

2.5 Antibodies

The antibodies used in this study are summarized in Table 2.1.

2.6 RNAi mediated silencing of Mst1 and Survivin

Stealth select RNAi™ for Mst1 and Survivin (both from Invitrogen, USA) were utilized to deplete Mst1 and Survivin protein level respectively. Mst1 silencing was carried out using 10 nM of the oligo (AAU GAU AUC AGA UAC AGA ACC AGC C). Survivin depletion on the other hand was performed using 10nM of the oligo (HSS141245). RNAi Negative Control Duplexes (Invitrogen, USA) served as negative control in each experimental set. The indicated RNAi were introduced into the cells by transfection using Lipofectamine RNAiMAX (Invitrogen, USA). The transfected cells were then harvested 24 hours later and resuspended in 2x SDS sample buffer and boiled. Depletion was assessed by immunoblotting with the relevant antibodies.

Table 2.1. Antibodies used in this study

Antibody	Host	Application	Dilution	Immunogen	Company
Actin pAb	Goat	WB	1:5000	C-terminus of human actin	Santa Cruz Biotechnology
Survivin pAb	Rabbit	WB, IF	WB: 1:1000 IF: 1:1000	E. coli-derived recombinant human Survivin amino acid 1-142	R&D Systems
Flag-M2 mAb	Mouse	WB, IP	WB: 1:1000 IP: 1:500	FLAG sequence at the N-terminus, Met-N-terminus or C-terminus of FLAG fusion proteins	Sigma-Aldrich
mAb414 mAb	Mouse	IF	IF: 1:5000	Repeated FXFG repeat sequence in nucleoporins including p62, p152 and p90 and other proteins.	Covance Research Products
MST-1 pAb	Rabbit	WB	WB: 1:2000	Amino-terminal region of human Mst1	Cell Signaling Technology
Ran pAb	Goat	WB, IF	WB: 1:1000 IF: 1:1000	Epitope mapping at the C-terminus of Ran of human origin	Santa Cruz Biotechnology
GCN5 mAb	Mouse	WB	1:1000	Amino terminus of human GCN5L2	Cell Signaling Technology
CRM-1 mAb	Mouse	WB	1:1000	Human hCRM1 amino acid 2-122	BD Biosciences
Cleaved caspase-3 mAb	Rabbit	WB	1:1000	Amino-terminal residues adjacent Asp175 of human caspase-3	Cell Signaling Technology
Cleaved PARP mAb	Mouse	WB	1:1000	Carboxyl-terminal residues surrounding Asp214 of human PARP	Cell Signaling Technology
Cleaved DFF45	Rabbit	WB	1:1000	Carboxyl-terminal residues surrounding Asp224 of human DFF45	Cell Signaling Technology

Flag mAb	Rabbit	WB	1:1000	N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C	Sigma-Aldrich
GFP (B-2) mAb	Mouse	WB, IF	WB: 1:1000 IF: 1:1000	Full length GFP of Aequorea victoria origin	Santa Cruz Biotechnology
Myc mAb	Rabbit	WB, IF	WB: 1:1000 IF: 1:1000	Residues 410-419 of human c-Myc (EQKLISEEDL)	Cell Signaling Technology
α -tubulin mAb	Mouse	WB	1:1000	Carboxyl-terminal of α -tubulin	Sigma-Aldrich
Alexa Fluor 488 anti-rabbit IgG	Goat	IF	1:1000	Rabbit IgG heavy and light chains	Invitrogen
Alexa Fluor 594 anti-rabbit IgG	Goat	IF	1:1000	Rabbit IgG heavy and light chains	Invitrogen
Anti- α -tubulin clone DM1 α FITC-conjugate mAb	Mouse	IF	1:1000	Carboxyl-terminal of α -tubulin	Sigma-Aldrich
Anti-rabbit IgG HRP conjugate	Goat	WB	1:10,000	Rabbit IgG heavy and light chains	Dako
Anti-goat IgG HRP conjugate	Goat	WB	1:10,000	Goat IgG heavy and light chains	Dako

Abbreviations: IF, immunofluorescence; WB, western blot; IP, immunoprecipitation; pAb, polyclonal antibody; mAb, monoclonal antibody.

2.7 Molecular cloning

To create the mammalian expression plasmids, full-length Survivin was amplified (forward primer 5'-TAGGGCGGATCCAGATGGGTGCCCCGACGTTGC-3' and reverse primer 5'-TAGGGCGAATTCTCAATCCATGGCAGCCAGCTG-3') using HeLa cDNA library as template and Pfu polymerase (Invitrogen). The cDNA library was generated by reverse transcription-PCR from HeLa total RNA extracts. Survivin amplicons were then subcloned into the pcDNA3-FLAG vector using *Bam*HI and *Eco*RI sites or subcloned into pEF6/myc-His C vector using *Bam*HI and *Eco*RV restriction sites. GST-Survivin-GFP construct was generated by subcloning GST-Survivin amplicons into pEGFP-N3 vector using *Hind*III and *Eco*RI sites.

2.8 Time-Lapse imaging

Cells were seeded onto 60mm dishes and transfected with empty vector or GST-Survivin-GFP for 2 days. The transfected cells were then treated with or without VP16 and placed on a heat-controlled stage of a Zeiss Axiovert 200M microscope. The temperature was maintained at 37° and CO₂ levels were maintained at 5% using a CTI 3700 controller (Carl Zeiss). Phase contrast and fluorescent images were recorded (AxioCam camera and Axiovision 4.6 software) using a 20x objective.

2.9 Semi-quantitative reverse transcription-PCR

Total RNA was extracted from HeLa cells using the RNeasy kit (Qiagen, USA) according to the manufacturer protocol. Total RNA from non-treated and treated samples according to the time indicated were used for the preparation of first-strand cDNA by reverse transcription utilizing the MMLV-based reverse transcriptase purchased from Stratagene. Human survivin mRNA was amplified using the forward primer 5'-TAGGGCGAATTCATGGGTGCCCCGACGTTGC-3' and the reverse primer 5'-TAGGGCGGATCCTCAATCCATGGCAGCCAGCTG-3' (corresponding to full length survivin sequence). GAPDH was also amplified as an internal control. The

PCR cycling conditions were set to 25 rounds to prevent amplification beyond the exponential phase. Reaction products were analysed on 1.0% agarose gels containing ethidium bromide.

2.10 Quantitative Real-Time PCR

Primers were designed for Survivin, and GAPDH .To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between the two exons or in a different exon. The oligonucleotides were synthesized by Sigma (Sigma-Aldrich); Survivin forward primer: 5'-AGCCAAGAACAAAATTGCAAAGG - 3'; Survivin reverse primer: 5'-ATCCATGGCAGCCAGCTGCT - 3'; GAPDH forward primer: 5'-AAGGTGAAGGTCGGAGTCAAC - 3'; GAPDH reverse primer: 5'-GGGGTCATTGATGGCAACAATA - 3'. qRT-PCR of the two transcripts was performed using a StepOnePlus Real time PCR instrument (Applied Biosystems, USA). PCR amplifications were performed using KAPA SYBR FAST qPCR kit (KAPA Biosystems, USA) in a total volume of 20 µl, each reaction contained 2x Mastermix 10 µl, each primer 0.4 µl (10 µmol/l), cDNA 1 µl, ROX 0.4 µl and ddH₂O 7.8 µl. All samples were measured in triplicate. For all samples, positive and negative no template controls were performed.

2.11 Survivin stability assay

MG132 (Sigma-Aldrich) was used to accumulate a pool of endogenous Survivin proteins from Hela cells treated with or without VP16. The cells were then washed three times with PBS to lift the proteasome inhibitory effects of MG132. After MG132 washoff, the productions of new Survivin proteins were terminated by the addition of CHX (Sigma-Aldrich). SDS-PAGE and western blot were performed against Survivin for analysis and quantification at each indicated time points after MG132 release.

2.12 Caspase-3 activity assay and analyses

Nuclear-cytoplasmic fractions were prepared as described in section 2.4 from Hela cells transfected Control RNAi, Survivin RNAi or mock transfected, with or without VP16 treatment. Lysates were snap-frozen and stored at -80°C. 96-well microtiter plates (Nunc) were used for this assay together with the caspase-3 substrate, fluorogenic tetrapeptide Ac-DEVD-AMC (Sigma, USA). 5 ng of the substrate was incubated at room temperature for three hours in 200 µl of reaction buffer (20 mM HEPES, pH 7.4, with 2 mM EDTA, 0.1% CHAPS, and 5 mM DTT) and 10 µl of cytoplasmic extracts with or without 5µl of the corresponding nuclear extracts.

Spectrofluorometer was used to measure the production of fluorescence by substrate-cleavage, with excitation and emission wavelength set at 355 nm and 460 nm respectively. For each experimental set-up, results were expressed as the mean relative percentage of caspase-3 activity between samples containing only the cytoplasmic extracts and samples containing both cytoplasmic and nuclear extracts. For better and more accurate comparison, the read-outs obtained from cytoplasmic extract only samples were set as the absolute value (100%) such that the measurements from the corresponding samples containing both the cytoplasmic and nuclear extracts can be normalized accordingly.

CHAPTER 3 : RESULTS

3.1 HeLa cells can be stimulated to undergo apoptosis by low dose of VP16 and UV-irradiation.

I commenced the study by first establishing an experimental system in which apoptosis can be induced effectively without compromising potential complications in data collection later on. Since we seek to dissect out the physiological pathway of when and how the cells decide to commit to apoptosis, suitable choice of drugs and dosages are crucial to so called prolong the period of this decision-making timeframe for analysis purposes. It would be an arduous attempt to achieve our objectives if the cells are killed off *en masse* within a short period of time. Protein kinase inhibitors like Staurosporine has adversely severe effects such that by 6 hours the morphology of the cells changed/blebbed/shrunked so much so that no meaningful data could be obtained and evaluated.

To this end, VP16 (DNA damaging agent) or etoposide and UV-irradiation were opted in this study as apoptotic stimuli. As a semisynthetic derivative of podophyllotoxin, the 4'-demethylation and an engineered b-glycosidic moiety in 7'-position of VP16 effectively transform these substances into irreversibly potent DNA topoisomerase II inhibitors. VP16 primarily generates errors in the DNA replication process by forming a trimeric complex together with topoisomerase II and DNA, thus preventing the DNA strands from re-ligation. The assembly of the complex involves the -OH of VP16 at position 4' being bound to the nucleic acid's phosphate group at one end and the amidation with topoisomerase II through the carbonyl group at the other. As such, the interference with topoisomerase II activity increases the propensity of DNA breaks to occur [157]. Furthermore, VP16 could alternatively act via the initiation of E-ring metabolism, which subsequently generates catechols or ortho-quinones that inactivate the DNA by forming chemical adducts [158]. VP16 is usually used in chemotherapy as well as to induce apoptosis in various cell lines [159-161]. Nonetheless, for this study, I utilized a decreased dosage of VP16 yet still substantially capable of invoking apoptotic response in cells. UV-irradiation, on the hand, elicits apoptosis by generating DNA lesions (single and double-stranded breaks), reactive oxygen species, and formation of

DNA photoproducts which are cytotoxic to the cells [162]. Even in the case of UV irradiation, I reduced the regular dosage from 100Jm^{-2} to 50Jm^{-2} .

The cytotoxicity of the lowered $20\text{ }\mu\text{g/ml}$ of VP16 and 50Jm^{-2} of UV-irradiation was biochemically evaluated by probing for common activated apoptotic markers. I had collected VP16-treated and UV-irradiated cell lysates in different time points and probed with caspases, inhibitor of caspase activated DNase (ICAD), and PARP in order to examine the apoptosis status of the cells.

A

B

Figure 3.1.1 VP16 and UV-irradiation induce apoptosis in HeLa cells. Total HeLa cell lysates were collected at the stated time points after stimulation with indicated treatment. VP16-induced (A) and UV irradiation-induced (B) cell death as shown by immunoblots of cleaved caspase-3, cleaved PARP and cleaved ICAD. Actin serves as loading control.

As shown in Fig. 3.1.1, active cleaved form of executioner caspases-3 could be detected 24 hours after drug addition, and visibly observed 12 hours post UV irradiation. In addition, Poly (ADP-ribose) polymerase (PARP) and ICAD, both substrates of the activated caspases, were also found cleaved at corresponding time points. These data supports the notion that the cells are indeed dying upon exposure to apoptotic agents albeit at lowered dosages.

3.2 The anti-apoptotic protein Survivin is degraded during apoptosis.

While the molecular mechanism in determining the life and death of a cell remains ill defined, it is clearly associated with the balance struck by the interplay between positive anti-apoptotic factors and negative pro-apoptotic factors. We believed that in dying cells, the tilt in balance and the convergence of the proapoptotic factors could be due to the removal and/or inactivation of the prosurvival factors. Following that, we sought to characterize pro-survival proteins that might be affected in Hela cells induced to undergo apoptosis. Survivin is a suitable candidate not only because it is highly expressed in cancer cells but also as an IAP, it is also a vital modulator of apoptosis [14, 31, 57]. Western blot revealed a marked decrease of total endogenous Survivin level in a time-dependent manner (Fig. 3.2.1) upon the induction of apoptosis both by VP16 treatment and also UV-irradiation.

Figure 3.2.1 Apoptotic degradation of pro-survival protein Survivin. (A) Total cell lysates collected at the stated time points after VP16 treatment and immunoblotted with anti-Survivin and anti-actin (loading control). (B) Total cell lysates were collected at indicated time intervals after UV irradiation. Survivin and tubulin (loading control) were probed by immunoblotting.

It is possible that the decline of endogenous Survivin could be due to the decrease of Survivin mRNA transcripts, or the post-translational degradation of the protein or both. To address the likelihood of Survivin being regulated at the transcription level during apoptosis, we performed semi-quantitative reverse transcription-PCR. Using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, the level of Survivin mRNA appeared to be relatively stable and unaffected during apoptosis (Fig. 3.2.2). This result was corroborated by real time-qPCR analysis (Fig 3.2.3). Hence, this effectively rules out apoptotic down-regulation of Survivin mRNA transcription.

Figure 3.2.2 Survivin mRNA level is unaffected during apoptosis. Total RNAs isolated from Hela cells treated with or without VP16 at the indicated time points. Semi-quantitative reverse transcription-PCR analysis was then performed using GAPDH as internal control. PCR cycles were kept minimal (25 cycles) such that both the amplification of the internal control and Survivin do not surpass the exponential phase.

Figure 3.2.3 qPCR of Survivin transcripts analysis in control and apoptotic cells. (A) Amplification plot for Survivin and GAPDH (internal control) for untreated and VP16-treated cells. (B) Dissociation Curve for Survivin. (C) Dissociation Curve for GAPDH. (D) Tabulated relative amount of gene expression from the amplification plot. *2x* denotes two times the amount of template used. (E) Gel picture of the amplicons after qPCR. Survivin~102 bp, GAPDH ~ 107 bp.

Endogenous Survivin turnover has been shown to be modulated by the ubiquitin–proteasome proteolytic pathway [34]. To investigate whether Survivin is similarly subjected to ubiquitin-related degradation upon apoptosis induction, we performed Survivin stability assay as denoted by the schematic in Figure 3.2.4A. MG132 is used to block the proteosomal activity and cyclohexamide (CHX) inhibits the synthesis of new proteins. We found that endogenous Survivin proteins are degraded at a faster rate in dying cells as compared with control cells following the resumption of proteasome activity (Fig. 3.2.4B). Moreover, graphical representation (Fig 3.2.4C) clearly showed the prominent decrease in the stability of endogenous Survivin during apoptosis.

Figure 3.2.4 Cell death triggers ubiquitin-proteasome proteolysis of Survivin.

(A) Schematic representation of Survivin stability assay. (B) Experiments performed as shown by the schematics in (A). Endogenous Survivin level was probed at indicated time points after the resumption of the proteasome activity. Tubulin serves as loading control. (C) Quantified graphical data presented as mean $\pm$ S.D. (error bar) of three independent experiments.

3.3 Endogenous Survivin redistributes into the nucleus of apoptotic cells.

As Survivin undergoes ubiquitin-dependent proteolysis in the nucleus [35], it is possible that Survivin relocation occurs during the progression of apoptosis. To test out this idea, immunofluorescence staining was performed against Survivin and mAb414, marker for nuclear pore protein complexes (NPCs). Indeed, I found that endogenous Survivin redistributes from the cytoplasm into the nucleus of VP16-treated cells in a time-dependent manner (Fig 3.3.1A). The nuclear envelope appeared to be intact throughout the course of experiment, thus indicating that its function as a physical barrier is not compromised. NPC had been reported to be the substrates of caspases only during late apoptosis [146-148]. Furthermore, I can deduce that the cells were likely to be in the early stages of apoptosis considering the fact that membrane blebbing was not observed even after 24 hours of VP16 treatment (Fig. 3.5A), although cleaved caspase-3 was evidently detected (Fig. 3.1A).

To demonstrate that the relocation of Survivin is a widespread phenomenon of dying cells, we quantitate the relative percentage of populations of HeLa cells having distinctive Survivin localization. Consistently, control HeLa cells showed predominantly cytoplasmic (cytoplasmic > nuclear) localization of Survivin [13, 134], whereas the addition of VP16 triggered a significant increase in the population showing predominantly nuclear (nuclear > cytoplasmic) localization of Survivin (Fig. 3.3.1B), up ~80% (Student's t-test, $p < 0.001$).

Similarly, in UV-irradiated cells, Survivin was redirected into the nucleus as well (Fig. 3.3.2A). Albeit in a relatively lesser magnitude, a considerable upsurge in the proportion of HeLa cells showing predominantly nuclear localization (nuclear > cytoplasmic) of Survivin (Fig. 3.3.2B) was observed, up ~30% (Student's t-test, $p < 0.001$). Taken together, this suggests that endogenous Survivin redistributes into the nucleus during the early stages of apoptosis.

Figure 3.3.1 Apoptotic induction by VP16 causes the mislocalization of pro-survival protein Survivin in HeLa cells. (A) Survivin mislocalizes into the

nucleus after VP16 addition. Localization of nuclear pore complex and Survivin were visualized by immunostaining in the course of VP16 treatment. Cells were fixed and stained with anti-Survivin and mAb414 (marker for nuclear pore complex) after treatment with VP16 at indicated time points. *Images are acquired with auto-optimal exposure mode.* Scale bar indicates 10 μ m. (B) Population of Hela cells with predominantly nuclear Survivin increases drastically upon VP16 treatment. Cells incubated in the absence or presence of VP16 at the stated time points were fixed and stained with anti-Survivin. Data was quantified by counting the number of cells with predominantly nuclear Survivin, cells with predominantly cytoplasmic Survivin and cells with homogenous distribution of Survivin across the nucleocytoplasmic compartments. Results are expressed as percentage of populations of Hela cells having distinctive Survivin localization. These data represent the average of two independent experiments (n >250 cells).

Figure 3.3.2 Nuclear redistribution of Survivin upon UV-irradiation. (A) Cells were fixed and stained with anti-Survivin and mAb414 after UV-irradiation at indicated time points. Scale bar denotes 10 μ m. *Images are acquired with auto-optimal exposure mode.* (B) Quantification of cells with predominantly nuclear Survivin, predominantly cytoplasmic Survivin and cells with homogenous distribution of Survivin across the nucleocytoplasmic compartments. Results are presented as mean population percentage of HeLa cells having distinctive Survivin localization $\pm$ SD (error bar) of 3 independent experiments (n = 200 cells).

3.4 The nuclear accumulation of endogenous Survivin during the early stages of cell death is a result of RanGTP gradient breakdown.

The nucleocytoplasmic shuttling ability of Survivin is coordinated by the low molecular weight of Survivin, which allows passive diffusion, and also the presence of an intrinsic NES motif that directs Survivin export into the cytoplasm [31-33, 56]. Immunostaining of Survivin and mAb414 performed on Leptomycin B (potent CRM1 inhibitor) or VP16 treated Hela cells showed analogous nuclear localization of endogenous Survivin as compared to control (Fig. 3.4.1A), hinting a possible error along RanGTP-CRM1-Survivin axis.

Given the importance of the RanGTP gradient in nucleocytoplasmic trafficking, we hypothesized that the nuclear export pathway could similarly be affected when the RanGTP gradient collapsed upon apoptosis induction by VP16. Following that, FRET experiments were performed on Rango transfected Hela cells treated with or without VP16 for 24 hours. The biosensor Rango comprises of a normally extended conformation of CFP and YFP due to the binding of importin β in the absence of RanGTP, thereby inhibiting the occurrence of FRET. However, when RanGTP is present, importin β is released from the IBB (importin beta binding) domain, thus allowing the donor-acceptor pair to come into proximity, interact and give rise to FRET [163].

Using the acceptor photobleaching method, nuclear RanGTP level was then measured as the relative percentage of FRET efficiency. Treatment of VP16 causes considerable reduction of the RanGTP level in the nucleus of Hela cells (Fig. 3.4.1 B and C), down by almost half ($p < 0.001$ Student's t-test).

Figure 3.4.1 The collapse of RanGTP gradient is responsible for Survivin mislocalization during the early stages of VP16-induced apoptosis. (A) Immunostaining of non-treated, and treated Hela cells with LMB or VP16 against anti-Survivin and mAb414. (B) FRET analysis of the nuclear RanGTP gradient by Rango biosensor (fusion protein of CFP and YFP flanking an importin binding domain) was carried out on transfected Hela cells in the absence and presence of VP16. Rango undergoes FRET in the presence of RanGTP. Representative images as in (B) and quantified data (C) presented as mean±S.D. (error bar) of three independent experiments (n=120). *p<0.001 (Student's t-test).

The FRET analyses were then repeated for cells irradiated with UV for 12 hours and 24 hours respectively. Likewise, UV-induced apoptosis implicate abrogated RanGTP gradient across the nucleocytoplasmic compartment (Fig 3.4.2). Hence, we have consistently showed that apoptosis induction results in a significant reduction of nuclear RanGTP level in Hela cells.

Figure 3.4.2 UV-irradiation leads to the depletion of nuclear RanGTP level. FRET analysis of the nuclear RanGTP gradient was carried out on Rango-transfected Hela cells with or without UV-irradiation. Quantified data presented as mean $\pm$ SD (error bar) of 3 independent experiments (n=120). * denotes $p < 0.001$ (Student's t-test).

While most NES by themselves have low affinity for the binding of CRM1 [137], the involvement of RanGTP in the formation of the trimeric export complex is necessary for efficient cargo export. It has even been considered that the RanGTP gradient, set up by RCC1 activity in the nucleus and the cytoplasmic RanGAP activity, represents the prominent driving force behind the transport of nuclear protein [141]. Hence, we postulated that the low level of RanGTP in the nucleus during the early phases of apoptosis prevents the assembly of CRM1-RanGTP-Survivin trimeric export complex. To investigate the interaction between

the NES-containing Survivin and CRM1, Flag-tagged Survivin plasmid construct was first generated.

Next, co-immunoprecipitation was carried out using anti-Flag antibody on stably transfected (with Flag Survivin) HeLa cells with or without VP16 treatment. While the western blot analysis revealed that CRM1 co-immunoprecipitated with Flag-Survivin, interaction between CRM1 and Flag-Survivin was substantially weaker in samples treated with VP16 (Fig. 3.4.3). The data obtained precisely fits the model of co-operative binding between CRM1, RanGTP together with the NES-cargo as the basis of the trimeric export complex assembly [138, 164]. In correlation, VP16-induced decline of nuclear RanGTP level reduces the efficiency of RanGTP-CRM1-Survivin complex formation and thus leads to diminished export. All in all, this is a clear indication that the accumulation of Survivin in the nucleus is a result of nuclear export deregulation via the RanGTP-CRM1 pathway.

Figure 3.4.3 VP16-induced redistribution of Survivin to the nucleus is a result of nuclear export deregulation via the RanGTP-CRM1 pathway (A) Flag-Survivin was overexpressed, and immunoprecipitated with anti-Flag from cells incubated with or without VP16 for 24 hours. (B) Quantified Crm1 intensities normalized against Flag presented as relative fold change $\pm$ S.D. (error bar) of three independent experiments.

3.5 Nuclear relocalization and degradation of Survivin as a recurring apoptotic theme in H1299 cells

In vitro cell culture studies have shown that various cancer cell lines exhibit different cell fates in response to genotoxic stress. To substantiate our findings, we extended the experimental analyses to another available cell line expressing high levels of Survivin; H1299 cells. Indeed, upon exposure to VP16, Survivin was markedly degraded in a time-dependent manner (Fig. 3.5.1A). Immunofluorescence staining also demonstrated that the proportion of H1299 cells displaying predominantly nuclear localization of Survivin was approximately 50% more ($p < 0.001$ Student's t-test), when apoptosis was induced (Fig. 3.5.1B and C). In line, treatment of VP16 invoked significant reduction ($p < 0.001$ Student's t-test) of the RanGTP level in the nucleus of H1299 cells (Fig. 3.5.1D). As such, these results suggest that the nuclear-targeted degradation of Survivin is a common feature of apoptosis, at least in non-functional p53 cell lines that we had tested so far.

Figure 3.5.1 Endogenous Survivin is degraded and relocated to the nucleus of apoptotic H1299 cells (A) Total endogenous Survivin level depletes throughout the duration of VP16 treatment. (B and C) H1299 cells treated with or without VP16 were immunostained and quantified for the localization of Survivin across the nucleocytoplasmic compartments. Scale bar indicates 10 μ m. *Images are acquired with auto-optimal exposure mode.* (D) FRET analysis demonstrates that nuclear RanGTP level decreases during VP16-induced apoptosis in H1299 cell. * denotes $p < 0.001$ (Student's t-test)

3.6 Re-establishment of RanGTP gradient rescues apoptotic Survivin redistribution.

We speculated the possibility that the export of endogenous Survivin into the cytoplasm could resume if the RanGTP gradient recovers. In our earlier report, we have established the use of *MstI* siRNA to restore diminished nuclear RanGTP level [155]. Hence, by employing the same strategy, we sought to find out whether Survivin could be export to the cytoplasm in the presence of apoptotic stimuli after *MstI* knockdown. Western blot was performed on mock-transfected cells and cells transfected with *MstI* siRNA or Control siRNA to verify the depletion of MST1 protein level. Treatment of *MstI* siRNA achieved ~80% knockdown efficiency but does not affect Survivin and Ran (Fig. 3.6.1A). Consistently, the depletion of MST1 restores nuclear RanGTP in cells treated with VP16 (Fig. 3.6.1B).

A clear indication of RanGTP gradient collapse is the mislocalization of Ran from predominantly nuclear to being dispersed across the nucleocytoplasmic compartment. As shown in Figure 3.6.2, in accordance to previous studies [150, 165, 166], immunostaining showed that Ran was mislocalized to the cytoplasm in cells both exposed to VP16 and UV-irradiation, whereas Ran was mainly localized in the nucleus of control cells. *MstI* knockdown restores the nuclear localization of Ran in cells induced to undergo apoptosis.

Immunostaining was then carried out on cells transfected with *MstI* siRNA or Control siRNA, in the absence and presence of VP16. HeLa cells transfected with *MstI* siRNA demonstrated cytoplasmic localization of Survivin in apoptotic cells (Fig. 3.6.1C). This evidently indicates that restoration of the RanGTP gradient reinstates the export of Survivin into the cytoplasm of HeLa cells undergoing apoptosis.

Figure 3.6.1 Restoration of RanGTP gradient reinstates Survivin export. (A) Immunoblotting against Mst1, Survivin, Ran and Actin was performed on lysates collected from mock, Control siRNA or Mst1 siRNA-transfected HeLa cells. (B) Rango FRET analysis was performed on Mst1 siRNA or Control siRNA-transfected HeLa cells, with or without VP16 treatment. Quantified data presented as mean±S.D. (error bar) of three independent experiments (n=120). **p<0.001 (Student's t-test) (C) HeLa cells transfected with Mst1 siRNA or Control siRNA before 24 hours incubation with VP16. The cells were fixed and stained with anti-Survivin and mAb414. Scale bar denotes 10 μ m. *Images acquired with auto-optimal exposure mode.*

Figure 3.6.2 RanGTP gradient restoration rescues apoptotic mislocalization of Ran. (A) siRNA knockdown of Mst1 restored nuclear localization of Ran in VP16 treated cells. HeLa cells were transfected with Mst1 or control siRNAs for 48 hours. The transfected cells were then incubated with VP16 for another 24 hours followed by immunofluorescence staining using antibody against Ran and mAb414. (B) siRNA knockdown of Mst1 restored nuclear localization of Ran in UV-irradiated cells. Experiment was repeated as in (A) using UV-irradiation as apoptotic stimuli instead of VP16. Scale bars denote 10 μ m.

3.7 Ubiquitinated Survivin are retained in the nucleus pending proteolysis when nuclear export fails during early apoptosis.

To verify that the export deregulated Survivin was post-translationally degraded in the nucleus, CHX was used to block protein synthesis. As shown in Figure 3.7.1, treatment of CHX in the absence or presence of VP16 exhibited a steady decline in endogenous Survivin level for both the nuclear and also the cytoplasmic fractions. As the Survivin mRNA transcripts are relatively unaffected by VP16 as previously shown (Fig. 3.2.2), this data indicated that existing Survivin was actively being degraded when the protein translation process was inhibited. Treatment of proteasome inhibitor MG132 restored both the VP16-treated or non-treated endogenous Survivin protein level over time, particularly in the nuclear fractions (Fig. 3.7.1). Western blot illustrated that while Survivin level is stabilized in the cytoplasmic fraction, there is a marked increase of Survivin level in the nuclear fraction over time. More importantly exposure to MG132 demonstrated a greater restoration of nuclear Survivin in samples treated with VP16. This suggested that apoptosis accelerates the depletion of prosurvival factor Survivin in the nucleus.

Figure 3.7.1 Mislocalized Survivin in the nucleus are degraded by the ubiquitin-proteasome proteolytic pathway. (A) HeLa cells were treated with cyclohexamide and the nuclear-cytoplasmic fractions were collected after the indicated time points. Western Blot analysis was carried out against Survivin, α -tubulin and GCN5. α -tubulin and GCN5 served as loading controls and indicators for cytoplasmic and nuclear fractions respectively. Similar experiment was also performed with the exception that HeLa cells were incubated with cyclohexamide (CHX) and VP16 simultaneously. The decline of endogenous Survivin levels both in the cytoplasmic and nuclear fractions in the presence of cyclohexamide (with or without VP16) implies that existing Survivin protein is actively being degraded over time. (B) Incubation of HeLa cells in MG132 with or without VP16 demonstrated that the proteasome inhibitor MG132 stabilized endogenous Survivin level and more specifically prevented its degradation in the nucleus. Western blot analysis clearly illustrated that while Survivin level is stabilized in the cytoplasmic fraction, there is a marked increase of Survivin level in the nuclear fraction over time. Note that the increase in nuclear Survivin level is higher in HeLa cells treated both with MG132 and VP16.

The collapse in RanGTP gradient and hence the failed active transport system during the progression of apoptosis could not fully explain the redistribution of Survivin into the nucleus. Even as the active transport processes ceases, Survivin (16.5 kDa) can readily diffuse across the nuclear membrane until equilibrium is reached between the cytoplasm and the nucleus. However, Survivin is predominantly compartmentalized within the nucleus during apoptosis (Fig. 3.3.1 and Fig. 3.3.2). In fact, for both cases, the fraction of cell population displaying homogeneous distribution of Survivin across the nucleocytoplasmic boundary either decreases or remains unaffected after apoptosis is induced. This indicates that Survivin is being sequestered in the nucleus after the export system shuts down.

Upon further inspection, the Survivin Stability Assay suggested that ubiquitinated Survivin in the nucleus could lead to possible increase in sizes due to multiple conjugations of ubiquitin molecules. NPCs function as selectivity filters that allow molecules <40 kDa to traverse across the nuclear envelope. We reasoned that after the nuclear transport machinery fails during apoptosis, ubiquitinated Survivin molecules in the nucleus are prevented from diffusing out due to their increased effective molecular weight >40 kDa. Western blot showed that apoptotic samples co-immunoprecipitated greater abundance of ubiquitinated Survivin proteins at various molecular weights above 40 kDa (Fig. 3.7.2). Thus, apoptotic relocation of Survivin into the nucleus resulted in multiple ubiquitination of Survivin that increases its molecular weight up to >100 kDa, thereby preventing diffusion across the NPC after nuclear export is impeded.

Figure 3.7.2 Apoptosis induction resulted in enhanced poly-ubiquitination of Survivin. Survivin-WT-Myc was overexpressed and immunoprecipitated with anti-Myc antibody from Hela cells incubated with or without VP16 for 24 hours. The immunoprecipitated materials were immunoblotted against Survivin and ubiquitin.

3.8 Survivin cannot inhibit apoptosis when compartmentalized in the nucleus.

During the early stages of apoptosis, Survivin relocates from the cytoplasm into the nucleus (Fig 3.8.1A) due to the collapse of RanGTP gradient whereas both the pro-caspases and activated cleaved caspase-3 remains in the cytoplasm (Fig. 3.8.1B and C). We reasoned that the apoptotic accumulation of Survivin in the nucleus prevents it from interacting with its target proteins in the cytoplasm. As such, the cytoprotectivity of Survivin is compromised even before it is degraded.

A **B** **C**

Figure 3.8.1 Localization of Survivin and activated Caspase-3 during the initial stages of apoptosis. (A) Nuclearcytoplasmic fractions collected from VP16-treated cells at the indicated time points. The samples were immunoblotted against anti-Survivin, anti-tubulin and anti-GCN5. (B) Procasase-3 and cleaved caspase-3 are predominantly cytoplasmic in VP16-treated cells. (C) Immunostaining of Survivin and caspase-3 was performed on apoptotic Hela cells. Activated cleaved caspase-3 did not co-localize with Survivin during apoptosis. Addition of VP16 relocates Survivin to the nucleus whereas activated caspase-3 was found to be predominantly cytoplasmic. Scale bar indicates 10 μ m.

To study the impact of nuclear Survivin accumulation on apoptosis, we performed the caspase-3 activity assay using the cleavage of caspase-3 substrate (Ac-DEVD-AMC) as fluorogenic reporter. Instead of using total lysates to probe for caspase-3 activity, the cytoplasmic fractions were used in line with prevalence of caspase-3 in the cytoplasm after apoptosis induction consistent with various reports that demonstrated caspase-3 only enters the nucleus during late apoptosis when the nuclear envelope breaks down. Since Survivin redistributes into the nucleus during apoptosis, parallel experiments were set up involving a portion of the nuclear fraction being added back to the corresponding cytoplasmic fraction before caspase-3 activity readout was taken. As shown in Figure 3.8.3, the addition of Survivin-containing nuclear fractions back its corresponding cytoplasmic fractions yielded significantly lower caspase-3 activity.

Next, in order to verify that the caspase-3 inhibitory effect was due to the presence of Survivin in the nuclear fraction, the assay was performed on Hela cells transfected with *Survivin* siRNA. 12 pmol of *Survivin* siRNA achieved ~90% knockdown efficiency of the Survivin protein level (Fig 3.8.2). The assay revealed that nuclear fractions depleted of Survivin had little effect on VP16-induced caspase-3 activity (Fig. 3.8.3). In other words, the knockdown of Survivin abolishes the suppressive effect of the supplemented nuclear fraction on cytoplasmic caspase-3 activity, thus certifying that the confinement of Survivin in the nucleus reduces its cytoprotectivity.

Figure 3.8.2 siRNA knockdown of Survivin. (A) Survivin depletion from HeLa cells by 24 hours Survivin RNAi transfection and immunoblotted against Survivin and Actin. (B) HeLa cells were transfected with Survivin siRNA or Control siRNA before incubation with VP16 for 24 hours. Cells were fixed and stained with anti-Survivin and mAb414. Scale bar indicates 10 μ m.

Figure 3.8.3 The cytoprotective activity of Survivin is inhibited by apoptotic nuclear compartmentalization. Caspase-3 activity assay. Each experiment was performed in triplicates in three independent experiments. Data presented as mean±S.D. (error bar). *p<0.001 (Student's t-test).

This data corroborated other reports that had demonstrated a link between cell death and the nuclear enforcement of Survivin by NES-mutation experiments or the expression of NLS- Survivin fusion proteins [35, 55, 57]. Additionally, transfection of Hela cells with GST-Survivin-GFP showed that the mutant Survivin is retained in the cytoplasm due to its increased size (~70 kDa) from passing through the NPC and reduces the percentage of apoptotic cells in the presence of apoptotic stimuli (Fig. 3.8.4). In summary, the anti-apoptotic activity of Survivin is abrogated when it is compartmentalized in the nucleus during the progression of apoptosis.

Figure 3.8.4 Nuclear precluded mutant GST-Survivin-GFP reduces the apoptotic effect of VP16 (A) Expression of mutant GST-Survivin-GFP in transfected HeLa cells. (B) GST-Survivin-GFP remains in the cytoplasm even after prolonged VP16 treatment. Time-lapse analyses of vector or GST-Survivin-GFP transfected HeLa cells in the presence of VP16. Scale bar indicates 10 μ m. (C) Cytoplasmic retention of mutant GST-Survivin-GFP partially rescues VP16-induced cell death. HeLa cells transfected with vector or GST-Survivin-GFP for 2 days before VP16 treatment. The cells were then collected after 40 hours and stained with trypan blue. Percentage of cell death was expressed as death index plotted against time. * $p=0.0014$ (Student's t-test).

CHAPTER 4 : DISCUSSION

4.1 Survivin withdrawal via nuclear export impairment commits cells to apoptosis.

Apoptosis is a process whereby cells play an active role leading to their own demise (cell suicide). The survival factors function by upregulating the expression and/or activity of anti-apoptotic regulatory molecules and maintain the dormancy of apoptotic executors. Upsetting this balance will trigger the cells to initiate the execution of apoptosis. Hence, it is tempting to speculate on the role of the cell particularly pertaining to the fate of existing pro-survival factors and the molecular events that leads to its subsequent cell death. In this study, we have reported a novel regulatory pathway mediating the active removal of prevailing pro-survival protein Survivin during the onset of apoptosis by the failed nuclear export machinery.

Survivin is an important bifunctional protein integral as a component of the Chromosome Passenger Complex and also participates in the negative regulation of apoptotic events. We have discovered that the pro-survival protein Survivin is actively being removed by the ubiquitin-proteasome proteolytic pathway upon the exposure to apoptotic stimuli. Moreover, Survivin redistributes from the cytoplasm to the nucleus during the progression of apoptosis. mAb414 staining indicates the preservation of the nuclear envelope integrity throughout the duration of the experiment, thus implying that the function of the nuclear membrane as a physical barrier was not compromised during the early stages of apoptosis.

This is indeed surprising considering the fact that the cytoplasmic localization of Survivin is necessary for its cytoprotective IAP function as shown by various reports [28, 29, 35]. For a cell under genotoxic stress, it is conceivable that the pro-survival factors will proceed and attempt to rescue the cell from dying. Then again, the cell will probably never die if the pro-survival factor continues to exist and function. Our data demonstrated that the relocation of endogenous Survivin into the nucleus is a widespread phenomenon during the initial phases of apoptosis.

The progressive accumulation of endogenous Survivin in the nucleus was found to be associated to the breakdown of the RanGTP gradient upon apoptosis

induction. As a small protein, Survivin is able to shuttle between nuclear and cytoplasmic compartments by passive diffusion under normal circumstances. Nonetheless, the presence of an active NES directs continuous export of Survivin into the cytoplasm via the interaction with its export receptor CRM1. While most NES by themselves have low affinity for the binding of CRM1 [137], the involvement of RanGTP in the formation of the trimeric export complex is necessary for efficient cargo export. Further investigations revealed the redistribution of endogenous Survivin in the nucleus is a consequence of deregulation along the RanGTP-CRM1 axis. The interaction between Survivin and CRM1 was found to be significantly weaker during early apoptosis. This precisely fits the model of co-operative binding between CRM1, RanGTP together with the NES-cargo as the basis of the functional trimeric export complex assembly [138, 164]. The decreased nuclear RanGTP level interferes the trimeric RanGTP-CRM1-Survivin complex formation and thus leads to diminished export. Conversely, the restoration of RanGTP gradient by *Mst1* knockdown reinstated the cytoplasmic localization of endogenous Survivin in apoptotic cells. Hence, we resolved the mechanism behind the failed nuclear export system during the initial phases of apoptosis.

In addition, failure to actively and rapidly transport Survivin back into the cytoplasm helps to justify its decreasing protein level in the course of apoptosis. The presence of Survivin in the nucleus promotes its clearance by the ubiquitin-proteasome proteolytic pathway. This corroborates previous findings, which collectively reported the reduced stability of Survivin in the nucleus as a result of proteasomal degradation [34, 35]. To this end, it has been reported that forcible targeting of Survivin into the nucleus using Survivin-NLS-GFP fusion protein accelerates its clearance by the ubiquitin-proteasome proteolytic pathway. In addition, when treated with Leptomycin B (CRM1 specific inhibitor), endogenous Survivin level was reduced significantly. Overall, it is rational to deduce that endogenous Survivin is vigorously targeted for ubiquitin-proteasomal mediated degradation in the nucleus during apoptosis.

More importantly, this implies that the cessation of nuclear export could be the regulatory mechanism for the intentional removal of pro-survival factor Survivin in cells committed to apoptosis. The conjugation of ubiquitin to Survivin also partially explains how Survivin is retained in the nucleus even after the nuclear export processes are impeded. The small molecular weight of Survivin should theoretically permit passive diffusion back into the cytoplasm even as the RanGTP gradient collapsed. We showed that apoptotic relocation of Survivin into the nucleus resulted in multiple ubiquitination of Survivin that effectively increases its molecular weight up to ~100kDa thereby prevents diffusion across the NPC.

Survivin has been demonstrated by numerous studies to directly and/or indirectly interact and thus regulate the activities of caspases [10-13]. During interphase, under normal circumstances, Survivin localizes predominantly in the cytoplasm. Therefore, it can interact and inhibit caspases that might be activated when the cell is under stress. However, this does not mean that caspase activity is only confined to the cytoplasm. Though the effector caspase-3 is found in apoptotic nuclei, this observation is commonly made during the later stages of apoptosis usually after the integrity of the nuclear membrane is compromised. In recent years, a flurry of studies has associated the nuclear Survivin to the sensitization of viable cells to apoptotic stimuli [28, 35, 57]. Although various strategies have been employed to force the entry of Survivin into the nucleus, the general consensus is that nuclear Survivin could no longer protect cells against apoptosis. Nonetheless, it still remains obscure whether it is indeed nuclear retention or merely mutant-specific effects being the causative factor of Survivin's inhibited cytoprotection. Here, we demonstrated that endogenous Survivin had limited cytoprotectivity in the nucleus as it was prevented from reaching and interacting with its protein targets (such as the caspases) in the cytoplasm during apoptosis. As described in the results section (Figure 5E), the addition of Survivin-containing nuclear fractions back its corresponding cytoplasmic fractions yielded significantly lower caspase-3 activity. It appears that the anti-apoptotic activity of Survivin is

abrogated when it is compartmentalized in the nucleus during the progression of apoptosis.

In sum, while we have previously reported the collapse of RanGTP gradient results in the shutting down of nuclear import processes during the early stages of apoptosis [155], we have shown here that the nuclear export machinery is simultaneously impaired as well. More importantly, our data indicates an unprecedented nucleocytoplasmic transport associated mechanism to regulate the removal of existing pro-survival factor for cell death to occur (Fig. 4.1). The abolished cytoprotectivity of Survivin couple to its degradation in the nucleus ensures active withdrawal of positive signals during the early stages of apoptosis. Consequently, the survival balance is tilted and the unrestricted, widespread activation of pro-apoptotic effector molecules will irreversibly contribute towards cell death by apoptosis. Given this new perspective on how nuclear transport actively regulates apoptosis, the work done in this study further advances our understanding in the field of apoptosis and potentially carries substantial implications in cancer therapy and drug development.

Figure 4.1 Hypothetical model of the implication of nuclear export impairment in cell death. Schematic depicts the role of nuclear export system in regulating the commitment of cells featuring the prosurvival protein Survivin during the onset of apoptosis. Conventionally, minimal and/or incidental occurrence of cell stress response is counteracted by the prevalence of abundant anti-apoptotic Survivin in the cytoplasm. However, once the nuclear transport processes shuts down, Survivin is unable to engage activated executioner molecules as it redistributes into the nucleus and undergoes rapid degradation. Thus, this lowers the apoptotic threshold thereby allowing the uninhibited accumulation of apoptotic effectors like caspase-3. As such, the apoptotic signals then further contributes to the irreversible cascade progression culminating in the death of the cell.

4.2 Emerging role of nucleocytoplasmic transport as active regulators of apoptosis.

As presented in the Introduction Section 1.7, it is clear that the nucleocytoplasmic transport system is invariably involved during the course of apoptosis, one way or the other. Much emphasis has been placed on its role in modulating apoptotic molecular mediators, from apoptotic kinases and effector molecules to the prosurvival factors *et cetera*, in terms of their placement within the cell to sustain the activated death signal necessary for apoptosis progression. Nonetheless, later studies attempting to better understand how the nucleocytoplasmic components responds to cell death triggers seemed to unravel another highly likely yet impactful possibility. A series of longstanding questions on why and how the trafficking machineries are affected as a consequence of apoptosis suddenly become what if these perturbed processes are the triggers themselves?

These publications appeared to advocate the concept that nuclear import impediment might serve as a trigger that sets off their own death pathway. The metastatic suppressor protein CC3 is known to interact favorably with components of the nuclear transport system, namely importin β , transportin, nucleoporins and exportin-4. It was reported that the high prevalence of endogenous CC3 and recombinant CC3 overexpression leads to a significant reduction of nuclear import processes and increased susceptibility to apoptosis [167]. In support, the effect of the dominant-negative mutant of importin β (45-462) is more forthcoming in that the expression of this mutant results in obstructed nuclear transport pathways causing cell death from apoptosis [167, 168]. Consistently, siRNA targeting of importin β and importin α substantially intensifies the proportion of apoptotic cells [169]. To an even further extent, aberrant manipulations of the distribution of importin via the overexpression of CAN/Nup 214, similarly demonstrated nuclear transport impairment, thus giving rise to elevated death index [170]. In accordance, we too have previously reported the phosphorylated-H2B death code linking RCC1-chromatin immobilization as being translated into the collapse of the RanGTP gradient. Subsequently nuclear import processes fails and leads to apoptosis [154].

While the premise of nuclear import breakdown as an active trigger of apoptosis emerge as an intriguing notion backed with solid data from various independent groups of researchers, as reviewed by Fahrenkrog, it is tempting to contemplate on the possibility of reduced nuclear export to induce apoptosis as well [149].

To our knowledge, this is the first study demonstrating the direct and detailed mechanism on nuclear export machinery in committing cells towards apoptosis. Taken together with our previous report, we proposed that the shutting down of both nuclear import and export machinery via the diminished RanGTP gradient may be important active processes that trigger the initiation and feedforward the progression of apoptosis. The abrogated activity of NF- κ B when it is retained in the cytoplasm coupled to the abolished cytoprotectivity of Survivin and its degradation in the nucleus ensures active withdrawal of positive signals during the initiation stages of apoptosis. Hence, the fate of the cell is sealed with rapid execution of the death sentence.

4.3 Current issues pertaining to Survivin-based therapies.

It was once predicted that the future of therapies targeting cancer would be exemplified by radical personalized diagnosis-treatments and the meticulous choice of tailor-suited single and/or combinatorial combative strategies. This was shed in the light of growing biological, genetic and physiological-interactive complexities of different forms and origins of cancer. In addition, there is also the emergence of escalating cases of therapeutic-resistant forms of cancers that seemed to be diversely affecting a subset of patients, hence the need of personalized medicine to better cater for and improve survival chances. Then Survivin came along and threw the entire field of oncology and pharmacology into frenzy. It is hardly a surprise considering the fact that Survivin is overexpressed in practically every human tumour examined. And unlike the other members of the IAP family, Survivin is only expressed in a small subset developmental-cell types under normal circumstances, whereas the rest of the IAPs are periodically found across non-cancerous cells of different origins. Armed with high potential promises,

accelerated advanced progress have been achieved in exploiting Survivin biology as novel cancer targeting strategies bypassing the agonizingly long timeline for the usual development of clinical agents [171, 172].

By interfering with Survivin's essential function both as a mitotic regulator and also cell death protector generally induces cell death. A plethora of antisurvivin therapeutics has been cleverly devised over the years to varied levels of success. One of the key focus areas involves the Antisense oligonucleotides (ASO)-based gene therapies. The efficacy of ASOs and siRNA based Survivin-targeting are firmly established particularly in the cell culture systems. Unfortunately, not only that were there adverse reactions, the ability to inhibit Survivin and slow tumour growth could not be replicated in animal studies. The poor outcomes were largely attributed to immune stimulations, non-specific targeting, and degradation of the active components by nucleases [173]. In addition, this is not a viable treatment method for brain tumours and brain metastases, as oligonucleotides could not cross the blood brain barrier.

Hence, current improvements involved enhanced delivery systems such as magnetic iron oxide nanoparticles harbouring membrane translocators conjugated to Survivin siRNA, liposome-mediated Survivin antisense delivery, antisurvivin amino silica nanoparticles, Locked-nucleic acid Survivin oligonucleotide and others [174-178]. From the pharmaceutical front, Isis Pharmaceuticals/Abbot Laboratories came up with ASO that is successful to induce widespread cell death in most tumour cell lines [49]. Eli Lilly Pharmaceuticals on the other hand, works with a modified Survivin targeting ASO (LY2181308) which is already advance in phase I clinical trials [179].

Another hopeful approach comes in the form of small-molecule suppressor of Survivin. Developed by Astellas Pharmaceuticals Incorporated, YM155 is a potent transcriptional repressor of Survivin and is capable of inducing apoptosis in diverse cancer cell lines and tumour xenografts [180]. Nevertheless, these results could not be replicated in clinical trials [181, 182]. In terms of immunotherapy, the basis revolves around the hypothesis that Survivin may be recognized as a non-self protein thereby eliciting immune response against Survivin-expressing cancer cells

[183]. Survivin based-primed dendritic cells or Survivin peptides are used in these therapies, however, only transient results were obtained in the clinical trials settings [184].

It is clear that anti-survivin therapy is highly relevant for the treatment of cancer. Nonetheless, there seems to be a translational barrier to reproduce the preclinical efficacy of antisurvivin therapeutics under clinical context. Alternatively, different anti-cancer strategies of inhibiting Survivin could be formulated. By exploiting the role of nucleocytoplasmic transport in regulating cell death, it is conceivable that one can screen for or design drugs and small molecules that specifically targets the binding of Survivin and Crm1, leading to its retention and autologous degradation in the nucleus. To achieve an optimum and thorough treatment strategy, a combinatorial therapy (alongside chemo- or radio- therapy) could then be devised to effectively induce apoptosis in the cancer cells.

It is apparent that further basic studies need to be done to comprehensively understand this enigmatic protein whose physiological functions lies between the crossroads of life and death. Undoubtedly, more questions have to be asked in the hope of obtaining answers that will bridge the gap between preclinical trials to effective translational bedside treatment.

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Survivin withdrawal by nuclear export failure as a physiological switch to commit cells to apoptosis

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Apoptosis is a tightly controlled process regulated by many signaling pathways; however, the mechanisms and cellular events that decide whether a cell lives or dies remain poorly understood. Here we showed that when a cell is under apoptotic stress, the prosurvival protein Survivin redistributes from the cytoplasm to the nucleus, thus acting as a physiological switch to commit the cell to apoptosis. The nuclear relocalization of Survivin is a result of inefficient assembly of functional RanGTP–CRM1–Survivin export complex due to apoptotic RanGTP gradient collapse. Subsequently, Survivin undergoes ubiquitination, which not only physically prevents its diffusion back to the cytoplasm but also facilitates its degradation. Together, this spatial and functional regulation of Survivin abolishes its cytoprotective effect toward the apoptotic executors and thus commits a cell to apoptosis. Our data indicate that the withdrawal of Survivin is a novel and active physiological regulatory mechanism that tilts the survival balance and promotes the progression of apoptosis.

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The significance of apoptosis is never undermined given its central role in normal development and homeostasis of multicellular organism. Apoptosis progression relies on the interplay between prosurvival factors and proapoptotic factors, which crucially decides whether a cell under stress lives or dies. The family of evolutionary conserved prosurvival proteins, inhibitor of apoptosis proteins (IAPs), is essential in protecting cells from entering apoptosis by negative regulation of downstream caspases.^{1–5} Survivin (16.5 kDa) is the smallest member of the IAP family containing a single baculovirus IAP repeat motif and it is frequently overexpressed and/or deregulated in most neoplastic tissues.^{6–10} This makes Survivin a leading research candidate in the development of prognostical tool and a prime target in cancer therapy.^{11,12}

As a member of the IAP, Survivin exhibits antiapoptotic characteristics. Elevated level of Survivin has been reported to confer radiation resistance, whereas artificial knockdown of Survivin sensitizes and renders cells more susceptible to irradiation-induced cell death.^{13,14} Survivin is able to inhibit the apoptotic executors; for example, caspases, due to its cytoplasmic localization. It is actively and rapidly exported into the cytoplasm, as Survivin contains a canonical nuclear export signal (NES) domain. The exclusion of Survivin from the nucleus is mediated by the classic nuclear export mechanism involving the interaction between the transport receptor chromosomal region maintenance-1 (CRM1) and Ran-guanosine 5'-triphosphate (RanGTP).^{15–17} On a different

note, forced entry of modified Survivin mutants into the nucleus by different groups has been associated to ubiquitination-mediated proteolysis by 26S proteasome.^{18–20}

Theoretically, the presence of prosurvival factors in the cell renders the cell resistant to apoptotic stimuli. Hence, it is conceivable that modulatory mechanisms are in place to negate the effect of the prosurvival proteins and to intensify the activation of proapoptotic proteins for the progression of apoptosis. However, the fate and functionalities of existing prosurvival factors and proteins are incompletely elucidated during cell death. The study herein describes a novel regulatory pathway serving as a physiological switch to mediate the removal of prevailing prosurvival protein Survivin during the onset of apoptosis by the impairment of nuclear export processes that actively contributes toward the eventual demise of the cell.

Results

Prosurvival protein Survivin is degraded during the initial stages of apoptosis. We believed that in dying cells, the tilt in balance and the convergence of the proapoptotic factors could be due to the removal and/or inactivation of the prosurvival factors. Thus, we sought to characterize prosurvival proteins that might be affected in apoptotic cells. Survivin is a suitable candidate not only because it is highly expressed in cancer cells but also as an IAP, it is also a vital

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Keywords: apoptosis; Survivin; RanGTP; nuclear transport; ubiquitination

Abbreviations: CFP, cyan fluorescence protein; CHX, cyclohexamide; CRM1, chromosomal region maintenance-1; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; RanGTP, Ran-guanosin 5'-triphosphate; IAP, inhibitor of apoptosis protein; MST1, mammalian STE20-like protein kinase 1; NES, nuclear export signal; NLS, nuclear localization signal; NPC, nuclear pore complex; XIAP, X-linked Inhibitor of apoptosis protein; YFP, yellow fluorescence protein

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modulator of apoptosis.^{5,15,21} Western blot revealed a marked decrease of total endogenous Survivin level in a time-dependent manner (Figure 1a and Supplementary Figure S1a) upon the induction of apoptosis (Figure 1b and Supplementary Figure S1b). It is possible that the decline of endogenous Survivin could be due to the decrease of Survivin mRNA transcripts, or the post-translational degradation of the protein or both. To address the likelihood of Survivin being regulated at the transcription level during apoptosis, we performed semiquantitative reverse transcription-PCR. Using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, the level of Survivin mRNA appeared to be relatively stable and unaffected during apoptosis (Figure 1c). Hence, this effectively rules out apoptotic downregulation of Survivin mRNA transcription.

Survivin turnover has been shown to be modulated by the ubiquitin–proteasome proteolytic pathway.²⁰ To investigate whether Survivin is similarly subjected to ubiquitin-related degradation upon apoptosis induction, we performed Survivin

stability assay as denoted by the schematics in Figure 1d. MG132 is used to block the proteosomal activity and cyclohexamide (CHX) inhibits the synthesis of new proteins. We found that endogenous Survivin proteins are degraded at a faster rate in dying cells as compared with control cells following the resumption of proteasome activity (Figure 1e). Moreover, graphical representation (Figure 1f) clearly showed the prominent decrease in the stability of endogenous Survivin during apoptosis.

Survivin redistributes into the nucleus of early apoptotic cells due to the collapse of RanGTP gradient. As Survivin undergoes ubiquitin-dependent proteolysis in the nucleus,¹⁹ it is possible that Survivin relocation occurs during the progression of apoptosis. To test out this idea, immunofluorescence staining was performed against Survivin and mAb414, marker for nuclear pore protein complexes (NPCs). Indeed, we found that endogenous Survivin redistributes from the cytoplasm into the nucleus of apoptotic cells in

Figure 1 Apoptotic degradation of prosurvival protein Survivin (a) Total cell lysates collected at the stated time points after VP16 treatment and immunoblotted with anti-Survivin and anti-actin (loading control). (b) VP16 treatment-induced cell death as shown by immunoblots of cleaved Caspase-3, cleaved PARP and cleaved ICAD. (c) Total RNAs isolated from HeLa cells treated with or without VP16 at the indicated time points. Semiquantitative reverse transcription-PCR analysis was then performed using GAPDH as internal control. (d) Schematic representation of Survivin stability assay. (e) Experiments performed as shown by the schematics in (d). Endogenous Survivin level was probed at indicated time points after the resumption of the proteasome activity. Actin as loading control. (f) Quantified graphical data presented as mean $\pm$ S.D. (error bar) of three independent experiments

Figure 2 RanGTP gradient collapse induces Survivin redistribution into the nucleus during apoptosis. (a) Survivin and mAb414 staining after VP16 treatment at indicated time points. Images acquired with auto-optimal exposure mode. (b) Quantification of cells with distinctive Survivin localization across the nucleocytoplasmic compartments. Results are presented as mean population percentage of HeLa cells having distinctive Survivin localization $\pm$ S.D. (error bar) of three independent experiments ($n > 300$ cells). (c) Immunostaining of non-treated, and treated HeLa cells with LMB or VP16 against Survivin and mAb414. (d) FRET analysis of the nuclear RanGTP gradient by Rango biosensor (fusion protein of CFP and YFP flanking an importin β binding domain). Rango undergoes FRET in the presence of RanGTP. Representative images (left) and quantified data (right) presented as mean $\pm$ S.D. (error bar) of three independent experiments ($n = 120$). $*P < 0.001$ (Student's *t*-test). (e) Flag-Survivin was overexpressed, immunoprecipitated with anti-Flag from cells incubated with or without VP16 for 24 h. Bars, 10 μ m. (right) Quantified Crm1 intensities normalized against Flag was presented as relative fold change $\pm$ S.D. (error bar) of three independent experiments

a time-dependent manner (Figure 2a and Supplementary Figure S2a). The nuclear envelope appeared to be intact throughout the course of experiment, thus indicating that its function as a physical barrier is not compromised. NPC has been reported to be the substrates of caspases only during late apoptosis.^{22,23} To demonstrate that the relocation of Survivin is a widespread phenomenon, we quantitate the relative percentage of populations of HeLa cells having distinctive Survivin localization. Consistently, control HeLa cells showed predominantly cytoplasmic (cytoplasmic > nuclear) localization of Survivin,^{4,24} whereas the induction of apoptosis triggered a significant increase (Student's *t*-test, $P < 0.001$) in the population showing predominantly nuclear (nuclear > cytoplasmic) localization of Survivin (Figure 2b and Supplementary Figure S2b). This suggests that

endogenous Survivin redistributes into the nucleus during the early stages of apoptosis.

The nucleocytoplasmic shuttling ability of Survivin is coordinated by passive diffusion and also the presence of an intrinsic NES motif that directs Survivin export into the cytoplasm by CRM1.^{15,16,18,21} Hence, normal circumstantial diffusion of Survivin into the nucleus would not lead to its degradation as it is actively and rapidly exported back into the cytoplasm. Immunostaining of Survivin and mAb414 performed on Leptomycin B (LMB) (potent CRM1 inhibitor) or VP16-treated HeLa cells showed analogous nuclear localization of endogenous Survivin as compared with control (Figure 2c), hinting a possible error along RanGTP–CRM1–Survivin axis. Given the importance of the RanGTP gradient in nucleocytoplasmic trafficking, we hypothesized that the

nuclear export pathway could similarly be affected when the RanGTP gradient collapsed upon apoptosis induction. Using the acceptor photobleaching method of fluorescence resonance energy transfer (FRET), the nuclear RanGTP level was measured as the relative percentage of FRET efficiency. We showed that apoptosis induction results in a significant reduction of nuclear RanGTP level in HeLa cells (Figure 2d and Supplementary Figure S3).

To investigate the interaction between Survivin and CRM1 during apoptosis, Flag-tagged Survivin was overexpressed and immunoprecipitated using anti-Flag. Our data revealed that the interaction between CRM1 and Flag-Survivin was substantially weaker in the presence of apoptotic stimuli (Figure 2e). This indicates that the apoptotic accumulation of nuclear Survivin is driven by nuclear export deregulation by the RanGTP-CRM1 pathway.

Re-establishment of RanGTP gradient rescues Survivin redistribution. We speculated the possibility that the export of endogenous Survivin into the cytoplasm could resume if the RanGTP gradient recovers. In our earlier report, we have established the use of *mammalian STE20-like protein kinase 1* (*Mst1*) siRNA to restore diminished nuclear RanGTP level.²⁵ Hence, by using the same strategy, we sought to find out whether Survivin could be exported to the cytoplasm in the presence of apoptotic stimuli after *Mst1* knockdown. Western blot was performed on mock-transfected cells and cells transfected with *Mst1* siRNA or control siRNA to verify the depletion of MST1 protein level. Treatment of *Mst1* siRNA achieved ~80% knockdown efficiency, but does not affect Survivin and Ran (Figure 3a). Consistently, the depletion of MST1 restores nuclear RanGTP in cells treated

with VP16 (Figure 3b). A clear indication of RanGTP gradient collapse is the mislocalization of Ran from predominantly nuclear to being dispersed across the nucleocytoplasmic compartment. As shown in Supplementary Figure S4a and b, *Mst1* knockdown restores the nuclear localization of Ran in cells induced to undergo apoptosis. Immunostaining was then carried out on cells transfected with *Mst1* siRNA or control siRNA, in the absence and presence of VP16. HeLa cells transfected with *Mst1* siRNA demonstrated cytoplasmic localization of Survivin in apoptotic cells (Figure 3c). This evidently indicates that restoration of the RanGTP gradient reinstates the export of Survivin into the cytoplasm of HeLa cells undergoing apoptosis.

Ubiquitinated Survivin are retained in the nucleus pending proteolysis when nuclear export fails during early apoptosis. To verify that the export deregulated Survivin was post-translationally degraded in the nucleus, CHX was used to block protein synthesis. As shown in Figure 4a, treatment of CHX in the absence or presence of VP16 exhibited a steady decline in endogenous Survivin level for both the nuclear and also the cytoplasmic fractions. As the Survivin mRNA transcripts are relatively unaffected by VP16 as previously shown (Figure 1b), this data indicated that existing Survivin was actively being degraded when the protein translation process was inhibited. Treatment of proteasome inhibitor MG132 restored both the VP16-treated or non-treated endogenous Survivin protein level over time, particularly in the nuclear fractions (Figure 4b). Western blot illustrated that while Survivin level is stabilized in the cytoplasmic fraction, there is a marked increase of Survivin level in the nuclear fraction over time. More importantly,

Figure 3 Restoration of RanGTP gradient reinstates Survivin export. (a) Immunoblotting against Mst1, Survivin, Ran and Actin was performed on lysates collected from mock, Control siRNA or *Mst1* siRNA-transfected HeLa cells. (b) Rango FRET analysis was performed on *Mst1* siRNA or Control siRNA-transfected HeLa cells, with or without VP16 treatment. Quantified data presented as mean $\pm$ S.D. (error bar) of three independent experiments ($n = 120$). $**P < 0.001$ (Student's *t*-test) (c) HeLa cells transfected with *Mst1* siRNA or Control siRNA before 24 h incubation with VP16. The cells were fixed and stained with anti-Survivin and mAb414. Bar, 10 μ m. Images acquired with auto-optimal exposure mode

Figure 4 Ubiquitinated Survivin is sequestered and degraded in the nucleus upon apoptosis induction. **(a)** Nuclearcytoplasmic fractions were collected from CHX-treated cells at the indicated time points. The experiment was repeated on HeLa cells incubated with CHX and VP16 simultaneously. The samples were immunoblotted against Survivin, tubulin and GCN5. **(b)** Experiment was repeated as in **(a)** with the exception that CHX is replaced by MG132. **(c)** Survivin-WT-Myc was overexpressed and immunoprecipitated with anti-Myc antibody from HeLa cells incubated with or without VP16 for 24 h. The immunoprecipitated materials were immunoblotted against Survivin and ubiquitin

exposure to MG132 demonstrated a greater restoration of nuclear Survivin in samples treated with VP16. This suggest that apoptosis accelerates the depletion of prosurvival factor Survivin in the nucleus.

The collapse in RanGTP gradient and hence the failed active transport system during the progression of apoptosis could not fully explain the redistribution of Survivin into the nucleus. Even as the active transport processes ceases, Survivin (16.5kDa) can readily diffuse across the nuclear membrane until equilibrium is reached between the cytoplasm and the nucleus. However, Survivin is predominantly compartmentalized within the nucleus during apoptosis (Figure 2a and Supplementary Figure S2a). This indicates that Survivin is being sequestered in the nucleus after the export system shuts down.

Upon further inspection, the Survivin stability assay suggested that ubiquitinated Survivin in the nucleus could lead to possible increase in sizes due to multiple conjugations of ubiquitin molecules. NPCs function as selectivity filters that allow molecules < 40kDa to traverse across the nuclear envelope. We reasoned that after the nuclear transport machinery fails during apoptosis, ubiquitinated Survivin molecules in the nucleus are prevented from diffusing out due to their increased effective molecular weight > 40kDa. Western blot showed that apoptotic samples co-immunoprecipitated greater abundance of ubiquitinated Survivin proteins at various molecular weights above 40 kDa (Figure 4c).

Thus, apoptotic relocation of Survivin into the nucleus resulted in multiple ubiquitination of Survivin that increases its molecular weight up to ~100kDa, thereby preventing diffusion across the NPC after nuclear export is impeded.

Survivin cannot prevent apoptosis when compartmentalized in the nucleus. During the early stages of apoptosis, Survivin relocates from the cytoplasm into the nucleus due the collapse of RanGTP gradient, whereas both the ProCaspase-3 and activated cleaved Caspase-3 remains in the cytoplasm (Figure 5a and b). We reasoned that the apoptotic accumulation of Survivin in the nucleus prevents it from interacting with its target proteins in the cytoplasm. As such, the cytoprotectivity of Survivin is compromised even before it is degraded. To study the impact of nuclear Survivin accumulation on apoptosis, we performed the Caspase-3 activity assay using the cleavage of Caspase-3 substrate (Ac-DEVD-AMC) as fluorogenic reporter. Instead of using total lysates to probe for Caspase-3 activity, the cytoplasmic fractions were used in line with prevalence of Caspase-3 in the cytoplasm after apoptosis induction consistent with various reports that demonstrated that Caspase-3 only enters the nucleus during late apoptosis when the nuclear envelope breaks down. As Survivin redistributes into the nucleus during apoptosis, parallel experiments were set up involving a portion of the nuclear fraction being added back to the corresponding cytoplasmic fraction before Caspase-3

Figure 5 Apoptotic nuclear compartmentalized Survivin has abolished cytoprotectivity. (a) Nuclearcytoplasmic fractions collected from VP16-treated cells at the indicated time points. The samples were immunoblotted against Survivin, tubulin and GCN5. (b) Procaspase-3 and cleaved Caspase-3 are predominantly cytoplasmic in VP16-treated cells indicating early apoptosis. (c) Survivin depletion from HeLa cells by 24 h *Survivin* RNAi transfection and immunoblotted against Survivin and Actin. (d) HeLa cells were transfected with *Survivin* siRNA or Control siRNA before incubation with VP16 for 24 h. Cells were fixed and stained with anti-Survivin and mAb414. Bar, 10 μ m. (e) Caspase-3 activity assay. Each experiment was performed in triplicates in three independent experiments. Data presented as mean $\pm$ S.D. (error bar). * $P < 0.001$ (Student's *t*-test). (f) Hypothetical model depicting the role of nuclear export system in regulating the commitment of cells during the onset of apoptosis

activity readout was taken. As shown in Figure 5e, the addition of Survivin-containing nuclear fractions back its corresponding cytoplasmic fractions yielded significantly lower Caspase-3 activity. To verify that the Caspase-3 inhibitory effect was due to the presence of Survivin in the nuclear fraction, the assay was performed on HeLa cells transfected with *Survivin* siRNA. Treatment of *Survivin* siRNA achieved ~90% knockdown efficiency of the Survivin protein level (Figure 5c and d). The assay revealed that nuclear fractions depleted of Survivin had little effect on VP16-induced Caspase-3 activity (Figure 5e). In other words, the knockdown of Survivin abolishes the suppressive effect of the supplemented nuclear fraction on cytoplasmic Caspase-3 activity, thus certifying that the confinement of Survivin in the nucleus reduces its cytoprotectivity. This data corroborated other reports that had demonstrated a link between cell death and the nuclear enforcement of Survivin by NES-mutation experiments or the expression of nuclear localization signal-Survivin fusion proteins.^{19,21,26} In addition, transfection of HeLa cells with GST-Survivin-green fluorescent protein (GFP) showed that

the mutant Survivin is retained in the cytoplasm due to its increased size from passing through the NPC and reduces the percentage of apoptotic cells in the presence of apoptotic stimuli (Supplementary Figure S5). In summary, the anti-apoptotic activity of Survivin is abrogated when it is compartmentalized in the nucleus during the progression of apoptosis.

Discussion

Apoptosis is a process whereby cells have an active role leading to their own demise (cell suicide). The survival factors function by upregulating the expression and/or activity of antiapoptotic regulatory molecules and maintain the dormancy of apoptotic executors. Upsetting this balance will trigger the cells to initiate the execution of apoptosis. Hence, it is tempting to speculate on the role of the cell, particularly pertaining to the fate of existing prosurvival factors and the molecular events that leads to its subsequent cell death. In this study, we have reported a novel regulatory pathway mediating the active removal of prevailing prosurvival protein

Survivin during the onset of apoptosis by the impairment of nuclear export machinery.

Survivin is an important bifunctional protein integral as a component of the chromosome passenger complex and also participates in the negative regulation of apoptotic events. We have discovered that the prosurvival protein Survivin is actively being removed by the ubiquitin–proteasome proteolytic pathway upon the exposure to apoptotic stimuli. Moreover, Survivin redistributes from the cytoplasm to the nucleus during the progression of apoptosis. mAb414 staining indicates the preservation of the nuclear envelope integrity throughout the duration of the experiment, thus implying that the function of the nuclear membrane as a selective physical barrier is not compromised.

For a cell under genotoxic stress, it is conceivable that the prosurvival factors will proceed and attempt to rescue the cell from dying. Then again, the cell will probably never die if the prosurvival factor continues to exist and function. Our data demonstrated that the relocation of endogenous Survivin into the nucleus of HeLa cells is a widespread phenomenon during the initial phases of apoptosis. The progressive accumulation of endogenous Survivin in the nucleus was found to be associated to the breakdown of the RanGTP gradient upon apoptosis induction. In addition, this apoptotic nuclear relocalization of Survivin is a recurring phenomenon as observed even in the H1299 cells (Supplementary Figure S6). As a small protein, Survivin is able to shuttle between nuclear and cytoplasmic compartments by passive diffusion under normal circumstances. Nonetheless, the presence of an active NES directs continuous export of Survivin into the cytoplasm by the interaction with its export receptor CRM1. Although most NES by themselves have low affinity for the binding of CRM1,²⁷ the involvement of RanGTP in the formation of the trimeric export complex is necessary for efficient cargo export. Further investigations revealed the redistribution of endogenous Survivin in the nucleus is a consequence of deregulation along the RanGTP–CRM1 axis. The interaction between Survivin and CRM1 was found to be significantly weaker during early apoptosis. This precisely fits the model of cooperative binding between CRM1, RanGTP and the NES-cargo as the basis of the functional trimeric export complex assembly.^{28,29} The decreased nuclear RanGTP level interferes the trimeric RanGTP–CRM1–Survivin complex formation and thus leads to diminished export. Conversely, the restoration of RanGTP gradient by *Mst1* knockdown reinstated the cytoplasmic localization of endogenous Survivin in apoptotic cells. Hence, we resolved the mechanism behind the failed nuclear export system during the initial phases of apoptosis.

In addition, failure to actively and rapidly transport Survivin back into the cytoplasm helps to justify its decreasing protein level in the course of apoptosis. The presence of Survivin in the nucleus promotes its clearance by the ubiquitin–proteasome proteolytic pathway. This corroborates previous findings, which collectively reported the reduced stability of Survivin in the nucleus as a result of proteasomal degradation.^{19,20} More importantly, this implies that the cessation of nuclear export could be the regulatory mechanism for the intentional removal of prosurvival factor Survivin for cells to commit to apoptosis. The conjugation of ubiquitin to Survivin

also partially explains how Survivin is retained in the nucleus even after the nuclear export processes are impeded. The small molecular weight of Survivin should theoretically permit passive diffusion back into the cytoplasm even as the RanGTP gradient collapsed. We showed that apoptotic relocation of Survivin into the nucleus resulted in multiple ubiquitination of Survivin that effectively increases its molecular weight up to ~100 kDa, thereby prevents diffusion across the NPC.

Survivin has been demonstrated by numerous studies to directly and/or indirectly (by X-linked Inhibitor of apoptosis protein or hepatitis B virus X-interacting protein) interact and thus regulate the activities of caspases.^{1–4,30} During interphase, under normal circumstances, Survivin localizes predominantly in the cytoplasm. Therefore, it can interact and inhibit caspases that might be activated when the cell is under stress. However, this does not mean that caspase activity is only confined to the cytoplasm. Although the effector Caspase-3 is found in apoptotic nuclei, this observation is commonly made during the later stages of apoptosis, usually after the integrity of the nuclear membrane is compromised. In recent years, a flurry of studies has associated the nuclear Survivin to the sensitization of viable cells to apoptotic stimuli.^{9,19,21} Here, we demonstrated that endogenous Survivin had limited cytoprotectivity in the nucleus as it was prevented from reaching and interacting with its protein targets (such as the caspases) in the cytoplasm during apoptosis. As described in the results section (Figure 5e), the addition of Survivin-containing nuclear fractions back its corresponding cytoplasmic fractions yielded significantly lower Caspase-3 activity. It appears that the antiapoptotic activity of Survivin is abrogated when it is compartmentalized in the nucleus during the progression of apoptosis.

In conclusion, we have shown here that the nuclear export machinery is impaired. More importantly, our data indicate an unprecedented nuclearcytoplasmic transport associated mechanism to regulate the removal of existing prosurvival factor for cell death to occur (Figure 5f). The abolished cytoprotectivity of Survivin couple to its degradation in the nucleus ensures active withdrawal of positive signals during the early stages of apoptosis. This regulatory mechanism serves as a physiological decision switch, in which the survival balance is consequently tilted and the unrestricted, widespread activation of proapoptotic effector molecules will irreversibly contributes toward apoptosis.

Materials and Methods

Cell culture and transfection. HeLa cells were obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (Gibco, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Hyclone, Thermo Scientific, Logan, UT, USA) and 1% Penicillin/Streptomycin (Gibco, Invitrogen) at 37°C in a humidified incubator with 5% carbon dioxide. Transfections of Rango or Flag-Survivin into HeLa cells were carried out using Lipofectamine 2000, according to manufacturer's instructions (Invitrogen).

Immunofluorescence microscopy. Cells seeded onto 22 mm coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.5% Triton X-100 (USB, Cleveland, OH, USA) in PBS for 5 min. After washing three times with PBS, cells were blocked in 4% BSA (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature (RT) and then incubated with primary antibody diluted in TBST (50 mM Tris, pH7.6, 150 mM NaCl, 0.1% Tween-20) plus 4% BSA. Following washes with TBST, the cells were

incubated with appropriate secondary antibodies and incubated at RT for 1 h in the dark. Cells were then mounted onto glass slides using ProLong gold antifade reagent containing 4',6-Diamidino-2-phenylindole (Invitrogen). Images were acquired and analyzed using an Axiovert 200M inverted microscope (Carl Zeiss, Standort Gottingen, Vertrieb, Germany) and Axiovision 4.6 software. Unless specified, all images are acquired with fixed exposures.

Reagents. For VP16 treatment, 10 mg/ml VP16 (Sigma-Aldrich) in dimethyl sulfoxide (DMSO) stock was diluted in medium to a final concentration of 20 μ g/ml. MG132 and CHX (both from Sigma-Aldrich) were diluted with buffers to a final working concentration of 2 μ M and 20 μ g/ml, respectively. For LMB (Sigma-Aldrich) treatment, the stock solution of 5 μ g/ml in 100% ethanol was diluted with buffers to a final working concentration of 5 ng/ml.

Antibodies. The antibodies used were anti-Survivin rabbit polyclonal Ab (R&D Systems, Minneapolis, MN, USA); anti-actin goat polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mAb414 mouse monoclonal Ab (Covance Research Product, Princeton, NJ, USA); anti-Ran goat polyclonal Ab (Santa Cruz Biotechnology); anti-Caspase3 rabbit polyclonal Ab (Cell Signaling Technology, Beverly, MA, USA); anti-FLAG M2 mouse monoclonal Ab (Sigma-Aldrich); anti-GCN5 rabbit monoclonal Ab (Cell Signaling Technology); anti-Myc rabbit monoclonal Ab (Cell Signaling); anti-cmyc mouse monoclonal Ab (Santa Cruz Biotechnology); anti-Mst1 rabbit polyclonal Ab (Cell Signaling Technology); anti-Ran goat polyclonal Ab (Santa Cruz Biotechnology); anti-CRM1 mouse monoclonal Ab (BD Biosciences, San Jose, CA, USA); anti- α -tubulin mouse monoclonal (Sigma-Aldrich); anti-cleaved DFF45 (Asp224) rabbit polyclonal (Cell Signaling Technology); anti-cleaved PARP (Asp214) rabbit polyclonal (Cell Signaling Technology); Alex Fluor 488 goat anti-rabbit IgG (Invitrogen); Alex Fluor 594 goat anti-rabbit IgG (Invitrogen); and Alex Fluor 488 goat anti-mouse IgG (Invitrogen); horseradish peroxidase-conjugated anti-mouse IgG, anti-rabbit IgG and anti-goat IgG (Invitrogen).

Molecular cloning. To create the mammalian expression plasmids, full-length Survivin was amplified (forward primer 5'-TAGGGCGGATCCAGATGGGTGCCCGGACGTTGC-3' and reverse primer 5'-TAGGGCGAATTCTCAATCCATGGCAGCCAGCTG-3') using HeLa cDNA library as template and pfu polymerase (Invitrogen). The cDNA library was generated by reverse transcription-PCR from HeLa total RNA extracts. Survivin amplicons were then subcloned into the pcDNA3-FLAG vector using *Bam*H1 and *Eco*R1 sites or subcloned into pEF6/myc-His C vector using *Bam*H1 and *Eco*RV restriction sites. GST-Survivin-GFP construct was generated by subcloning GST-Survivin into pEGFP-N3 vector using *Hind*III and *Eco*R1 sites.

Time-Lapse imaging. Cells were seeded onto 60 mm dishes and transfected with empty vector or GST-Survivin-GFP for 2 days. The transfected cells were then treated with or without VP16 and placed on a heat-controlled stage of a Zeiss Axiovert 200M microscope. The temperature was maintained at 37°C and CO₂ levels were maintained at 5% using a CTI 3700 controller (Carl Zeiss). Phase contrast and fluorescent images were recorded (AxioCam camera and Axiovision 4.6 software, Carl Zeiss) using a 20 $\times$ objective.

FRET. Lab-Tek Chambered cover glasses (Nunc, Rochester, NY, USA) were used to seed the cells. FRET was then performed on a Zeiss LSM 510 Meta confocal microscope equipped with Incubator S (Pecon, Erbach, Germany). During the experiment, the temperature and CO₂ level was maintained at 37°C and 5%, respectively, by placing the seeded cells in Incubator S on the microscope stage. Acceptor Photobleaching method was used for FRET analysis of Rango biosensor.³¹ Rango consists of a cyan fluorescence protein (CFP) and a yellow fluorescence protein (YFP) domain flanking an importin- β binding domain. In brief, the biosensor will have FRET when RanGTP is present. Five consecutive images were obtained at 1% of the laser intensity in CFP and YFP channels before and after YFP photobleaching by repeatedly scanning the designated area at 75% laser intensity. The background CFP fluctuations during FRET were determined from the unbleached control cells present in the same field. FRET efficiency was calculated as $E_p\% = (I_0 - I_5) \times 100/I_0$, where I_5 and I_0 represent the CFP fluorescence intensity of the fifth and sixth images immediately before and after YFP photobleaching. At least 15 cells were analyzed for each experimental set. Results were presented as mean percentage FRET efficiencies $\pm$ S.D.

Preparation of cell extracts, immunoblotting and immunoprecipitation. For the collection of whole cell lysates, HeLa cells were

washed with PBS and lysed by M-PER (mammalian protein extraction reagent; Thermo Scientific-Pierce, Rockford, IL, USA) containing 10% Complete EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Schweiz, Switzerland) and 0.1% phosphatase cocktail inhibitors 1 and 2 (Sigma-Aldrich). Nuclear cytoplasmic fractions were prepared using NE-PER (nuclear and cytoplasmic extraction) (Thermo Scientific) according to manufacturer's protocol. $\times 2$ sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue) was added to whole cell lysates, nuclear lysates and cytoplasmic lysates before boiling the mixture for 10 min. Equal amounts of protein were resolved on SDS-polyacrylamide gel (PAGE) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 10% skim milk in TBST for 2 h at RT and probed with specific primary antibodies diluted in blocking solution at 4°C overnight. Membranes were washed thrice with TBST before incubating with horseradish peroxidase-conjugated secondary IgG antibodies (Invitrogen). After washing with TBST, immunoreactive proteins were detected by enhanced chemiluminescence (GE Healthcare-BioSciences, Piscataway, NJ, USA). For immunoprecipitation, HeLa cells stably transfected with Flag-Survivin or Survivin-Wt-Myc and HA-Ub were incubated with or without VP16 for 24 h. These cells were then lysed with M-PER and the cell lysates were incubated with the relevant antibody for 1 h at room temperature. Rec-Protein-G-Sepharose 4B (Invitrogen) were added to each experimental set. The resultant mixtures were further incubated for 1 h at room temperature before repeated washing with Wash buffer (TBS with 100 mM NaCl) to rid unbound proteins. Immunoprecipitated proteins were separated by SDS-PAGE before western blotting analyses.

RNAi-mediated silencing of Mst1 and Survivin. Stealth select RNAi for Mst1 and Survivin (both from Invitrogen) were used to deplete Mst1 and Survivin protein level, respectively, in HeLa cells. Mst1 silencing was carried out using 10 nM of the oligo (AAUGAUACAGAUACAGAACCCAGCC). Survivin depletion on the other hand was performed using 10 nM of the oligo (HSS141245). RNAi negative control duplexes (Invitrogen) served as negative control in each experimental set. The indicated RNAi were introduced into the cells by transfection using Lipofectamine RNAiMAX (Invitrogen). The transfected cells were then harvested 24 h later and resuspended in $\times 2$ SDS sample buffer and boiled. Depletion was assessed by immunoblotting with the relevant antibodies.

Semiquantitative reverse transcription-PCR. Total RNA was extracted from HeLa cells using the RNeasy kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer protocol. Total RNA from non-treated and treated samples according to the time indicated were used for the preparation of first-strand cDNA by reverse transcription using the MMLV-based reverse transcriptase (Stratagene, La Jolla, CA, USA). Human Survivin mRNA was amplified using the forward primer 5'-TAGGGCGAATTCATGGGTGCCCGGACGTTGC-3' and the reverse primer 5'-TAGGGCGGATCCCTCAATCCATGGCAGCAGCTG-3' (corresponding to full-length Survivin sequence). GAPDH was also amplified as an internal control. The PCR cycling conditions were set to 25 rounds to prevent amplification beyond the exponential phase. Reaction products were analyzed on 1.0% agarose gels containing ethidium bromide.

Survivin stability assay. MG132 (Sigma-Aldrich) was used to accumulate a pool of endogenous Survivin proteins from HeLa cells treated with or without VP16. The cells were then washed three times with PBS to lift the proteasome inhibitory effects of MG132. After MG132 washoff, the productions of new Survivin proteins were terminated by the addition of CHX (Sigma-Aldrich). SDS-PAGE and western blot were performed against Survivin for analysis and quantification at each indicated time points after MG132 release.

Caspase-3 activity assay and analyses. Nuclearcytoplasmic fractions were prepared as described above from HeLa cells transfected Control RNAi, Survivin RNAi or mock transfected, with or without VP16 treatment. Lysates were snap-frozen and stored at -80°C . Ninety-six-well microtiter plates (Nunc) were used for this assay together with the caspase-3 substrate, fluorogenic tetrapeptide Ac-DEVD-AMC (Sigma-Aldrich). A quantity of 5 ng of the substrate was incubated at 37°C for 3 h in 200 μ l of reaction buffer (20 mM HEPES, pH 7.4, with 2 mM EDTA, 0.1% CHAPS and 5 mM DTT) and 10 μ l of cytoplasmic extracts with or without 5 μ l of the corresponding nuclear extracts. Spectrofluorometer measured fluorescence produced by substrate cleavage with excitation and emission wavelength set at 355 and 460 nm, respectively. For each experimental set-up, results were expressed as the mean relative percentage of caspase-3 activity between samples

containing only the cytoplasmic extracts and samples containing both cytoplasmic and nuclear extracts. For better and more accurate comparison, the read-outs obtained from cytoplasmic extract only samples were set as the absolute value (100%), such that the read-outs from the corresponding samples containing both the cytoplasmic and nuclear extracts can be normalized accordingly.

Conflict of interest

The authors declare no conflict of interest.

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