# THE ANTI-APOPTOTIC FUNCTION OF HNRNP K IN HEPATOCELLULAR CARCINOMA CELL LINES

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**Declaration**

I hereby declare that this thesis is my original work

and it has been written by me in its entirety.

I have duly acknowledged all the sources

of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any

university previously.

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Xiao Ziwei

21 August 2012

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## SUMMARY

hnRNP K is a member of the large family of ubiquitously expressed heterogeneous nuclear ribonucleoproteins (hnRNPs), which are involved in a wide array of cellular processes. hnRNP K has been consistently reported to be upregulated in various cancers that include hepatocellular carcinoma (HCC), the third highest cause of cancer-related deaths globally, with apoptosis dysregulation as one of its hallmarks. hnRNP K has been implicated in apoptosis but its precise role in this process has not been clearly defined. Hence, this study aims to investigate the role of hnRNP K in the dysregulation of apoptosis in HCC.

Consistent with previous reports of hnRNP K upregulation in HCC, the results from this current study revealed that hnRNP K was detected abundantly in a panel of 17 HCC cell lines examined, suggesting its importance in HCC. 5-Fluorouracil (5-FU), a common anti-metabolite chemotherapeutic drug, was used to treat HCC cells and the experimental findings revealed that hnRNP K was downregulated independently of p53. Reduction of hnRNP K expression using specific siRNA was also shown to result in a decrease in HCC cell viability. The evidence for reduction in cell viability was demonstrated to be via apoptosis using various *in-vitro* functional analyses including TUNEL assay, PARP-1 cleavage and caspase 3/7 assay. In addition, when HepG2 cells (p53+/+) and Hep 3B cells (p53-null) were treated with hnRNP K siRNA to examine whether p53 plays a role in hnRNP K-induced apoptosis, their similar caspase-3/7 activity profiles suggested that the apoptosis induced was p53-independent.

To further investigate the underlying mechanism of hnRNP K-downregulation-induced apoptosis, hnRNP K siRNA treated cells were simultaneously treated with either caspase-8 or caspase-9 siRNA. Intriguingly, the results revealed that only the addition of caspase-8 siRNA was able to abrogate the increase in caspase-3/7 activity. Thus, this

indicated that hnRNP K-downregulation-induced apoptosis is mediated predominantly via the caspase-8 apoptotic signalling pathway.

A reduction in hnRNP K was also demonstrated to sensitise HCC cells towards apoptosis, where exposure of cells to TRAIL 24 hours post-treatment with hnRNP K siRNA significantly enhanced the potency of TRAIL. The enhanced caspase-3/7 activity was also observed to be independent of BID cleavage and revealed to be a direct effect of increased caspase-8 activity.

In an attempt to better understand this novel hnRNP K-caspase-8 pathway, exon array was performed on hnRNP K siRNA treated Hep 3B cells and the genes associated with caspase-8 function were selected for further analysis. Among the 109 genes evaluated, *TNFSF10* expression was strongly elevated while *BIRC3*, *CFLAR* and *XIAP* were distinctly downregulated. The expression levels of these 4 genes were also confirmed by real time PCR, which revealed that hnRNP K reduction causes a downregulation of anti-apoptotic genes *BIRC3*, *XIAP* and *CFLAR* at the mRNA level. Hence, the experimental findings suggested that hnRNP K exerts its anti-apoptotic effect by regulating the expression levels of various anti-apoptotic genes.

In conclusion, this study has established the anti-apoptotic role of hnRNP K in HCC with the use of HCC cell lines, which was shown to be mainly mediated via the upregulation of anti-apoptotic proteins that can inhibit caspase activity. Thus, the experimental findings from this study have provided a molecular basis for understanding hnRNP K's involvement in HCC, as well as aided in the identification of hnRNP K as a potential prognostic and therapeutic marker for HCC and other cancers.

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| | |
|---|---|
| APS | ammonium persulfate |
| Arg | arginine |
| °C | degree Celsius |
| 5-FU | 5’-Fluorouracil |
| bp | base pair |
| BSA | bovine serum albumin |
| cDNA | complementary DNA |
| CMV | cytomegalovirus |
| DAPI | 4',6-diamidino-2-phenylindole |
| DMEM | Dulbecco’s Modified Eagle’s Medium |
| DMSO | dimethysulfoxide |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| dNTP | 2’-deoxyrobonucleoside-5’-triphosphate |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediamine tetra-acetic acid |
| FACS | fluorescence-activated cell sorter |
| FasL | Fas-ligand |
| FBS | fetal bovine serum |
| FITC | fluorescein isothiocyanate |
| g | gram |
| gly | glycine |
| h | hour |
| HBV | hepatitis B virus |
| HCC | hepatocellular carcinoma |
| HCV | hepatitis C virus |
| hnRNP | heterogeneous nuclear ribonucleoprotein |
| hnRNP K | Heterogeneous nuclear ribonucleoprotein K |
| kDa | kilo Dalton (the unit of molecular mass) |
| KH | hnRNP K homology |
| KI | hnRNP K interactive |
| KNS | hnRNP K nuclear shuttling |
| LB | Luria bertani |
| LOH | loss of heterozygosity |
| mA | milliampere |
| MAPK | mitogen-activated protein kinase |
| mg | milligram |
| min | minute |
| ml | mililiter |
| mM | milimolar |

| | |
|---|---|
| mRNA | messenger RNA |
| NF-κB | Nuclear factor-kappa B |
| ng | nanogram |
| NLS | nuclear localisation signal |
| nm | nanometer |
| nM | nanomolar |
| NPC | nasopharyngeal carcinoma |
| OD | optical density |
| ORF | open reading frame |
| *p* | p-value |
| PAGE | polyacrylamide gel electrophoresis |
| PARP-1 | poly ADP-ribose polymerase |
| PBS | phosphate buffered saline |
| PCBP | poly (C) binding protein |
| PCR | polymerase chain reaction |
| PMSF | phenylmethysulfonyl fluoride |
| poly(C) | polycytosine |
| Pro | proline |
| PVDF | polyvinylidene difluoride |
| RhoD | rhodamine |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| rpm | revolutions per minute |
| RT-PCR | reverse-transcription polymerase chain reaction |
| sec | second |
| SD | standard deviation |
| SDS | sodium dodecyl sulphate |
| si-ctrl | non-specific control siRNA |
| siRNA | small interfering RNA |
| ssDNA | single stranded DNA |
| SV40 | Simian Virus 40 |
| tBID | truncated-BID |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| TUNEL | terminal deoxynucleotidyl transferase dUTP nick end labelling |
| U | unit of enzyme |
| UV | ultraviolet |
| V | Voltage |
| µg | microgram |
| µl | microliter |
| µM | micromolar |
| % (w/v) | gram per 100 milliliter |
| % (v/v) | milliliter per 100 milliliter |

# CHAPTER 1

## Introduction

## 1.1 Heterogeneous nuclear ribonucleoprotein K (hnRNP K)

### 1.1.1 hnRNPs and PCBPs

hnRNPs and functions

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a multifunctional protein that belongs to the subfamily of ubiquitously expressed heterogeneous nuclear ribonucleoproteins (hnRNPs). hnRNPs are predominantly nuclear RNA-binding proteins that associate with RNA polymerase II mRNA transcripts to form ribonucleoprotein (RNP) complexes, where processing of this subset of pre-mRNA into mature RNAs occurs (Dreyfuss et al., 1993). In addition, hnRNPs are among the most abundant nuclear proteins that form the major components of the nucleus when complexed to the pre-mRNAs (Chaudhury et al., 2010).

The hnRNP subfamily consist of major hnRNP proteins designated hnRNP A1 through hnRNP U, as well as other minor and less abundant hnRNP proteins (Dreyfuss et al., 2002). hnRNP A1, A2/B1, B2, C1 and C2 were first identified and termed the 'core' hnRNP proteins based on their strong association with pre-mRNAs (Beyer et al., 1977). As each hnRNP protein has a preferred nucleotide binding sequence and varies in abundance as well as post-translational modification, it has been hypothesized that a unique combination of hnRNP proteins forms for each pre-mRNA, dictated by the nucleotide binding sequence as well as the hnRNPs' relative expression levels (Chaudhury et al., 2010).

However, major hnRNP proteins may possess additional functional roles, given that they are present in abundance and are likely to be in excess over its respective binding sites (Chaudhury et al., 2010). In support of this, it was demonstrated that the hnRNP proteins are ubiquitously expressed in all tissue types at varying levels of abundance and that their relative ratios differ across different cell types (Kamma et al.,

1995). In addition, certain hnRNPs such as hnRNP K are not only localised in the nucleus, but also found to be present in the cytoplasm (Dreyfuss et al., 2002; Kamma et al., 1995). Indeed, apart from pre-mRNAs processing, e.g. splicing, hnRNP proteins can also exert an effect on other aspects of gene expression, which includes mRNA export, localisation, translation as well as mRNA stability (Dreyfuss et al., 2002). Besides affecting the metabolism of mRNA, hnRNP proteins' role in transcriptional regulation was also documented by Krecic and Swanson (1999). This was first demonstrated for hnRNP K that showed preferential binding to single stranded DNA (ssDNA) *in vitro* (Michelotti et al., 1996). Therefore, members of the hnRNPs, e.g. hnRNP K, are multifunctional proteins that can participate in a variety of cellular processes.

Poly(C)-Binding Proteins (PCBPs) and functions

Apart from being a member of the hnRNPs, hnRNP K also belongs to the group of poly(C)-binding proteins (PCBPs). RNA binding proteins are characterized based on their ability to bind to nucleic acid homopolymers, thus as its name suggests, PCBPs are able to bind to polycytosine, poly(C) with high sequence specificity and affinity. hnRNP K was the first among the hnRNPs to be identified to possess this capability to bind poly(C) (Swanson and Dreyfuss, 1988).

Of the five mammalian PCBPs, hnRNP K, PCBP1 and PCBP2 (also known as hnRNPE1 and hnRNPE2 respectively) have been studied in great detail, as compared to PCBP3 and PCBP4 (also known as MCG10) that were discovered in 2000 (Choi et al., 2009; Makeyev and Liebhaber, 2002). Based on amino acid sequence analyses, the PCBP gene family had evolved from a single originating gene to five separate and dispersed mammalian loci (Makeyev and Liebhaber, 2002). Hence, these PCBPs

share an overall structural anatomy that contains three hnRNP K homology (KH) domains (first identified in hnRNP K), which likely confer them with the ability to bind poly(C). Two of the KH domains are located near the N-terminal while the third domain can be found at the C-terminal of the proteins (Figure 1.1) (Makeyev and Liebhaber, 2002). PCBPs also contain the nuclear localisation signal (NLS) sequence that allows for their transport from the cytoplasm to the nucleus. Additionally, hnRNP K contains a hnRNP K-specific nuclear shuttling (KNS) domain between KH II and KH III domain that allows for bi-directional transport through the nuclear pore complex (Choi et al., 2009). Thus, PCBPs' ability to localize in both the cytoplasm and nucleus accounts for their diverse functions.

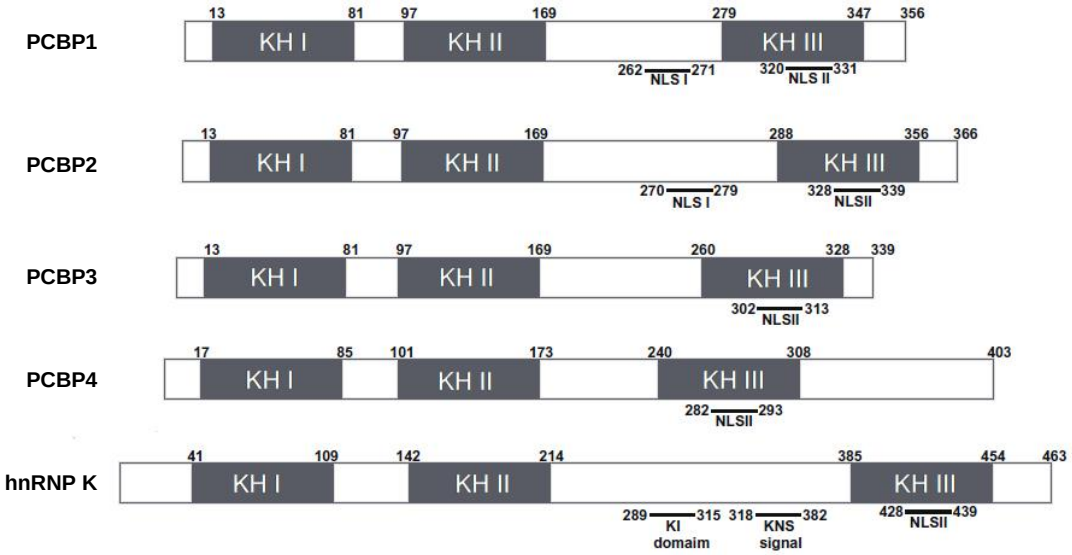


**Figure 1.1 Domain structure of PCBPs**
Members of the PCBP family are shown. Numbers shown indicate the respective amino acid residues from human sequence. KH (hnRNP K homology) domains I–III are shaded, and the NLS (nuclear localization sequence) signals are also indicated. hnRNP K also contains additional KI (hnRNP K interactive) domain as well as KNS (hnRNP K nuclear shuttling) signal (adapted from Choi et al., (2009) and reproduced with permission.)

Given their diverse functions, PCBPs have been reported to be involved in various post-transcriptional regulations, e.g. mRNA splicing, mRNA stabilisation as well as translational silencing or enhancement (Makeyev and Liebhaber, 2002). Notably, PCBPs have been implicated in the transcriptional regulation of genes due to their ability to bind poly(C) regions, of which hnRNP K has been the most frequently reported (Choi et al., 2009).

### 1.1.2 hnRNP K and its biological functions

Modular Structure of hnRNP K

Interest in hnRNP K grew in the early 1990s as it does not possess the RNA-binding domain that could be found in many of the other hnRNPs, but instead contained three repeats of KH domain that contain gly-arg-gly-gly (GRGG) sequences capable of binding RNA, single- and double-stranded DNA (Siomi et al., 1993). In addition, as many polypyrimidine tracts at the 3' ends of pre-mRNA introns were reported to be rich in cytidine, hnRNP K's ability to bind tenaciously to poly(C) garnered significant attention due to its possible role in mRNA splicing (Matunis et al., 1992).

Located on human chromosome 9q21.32-q21.33 (Tommerup and Leffers, 1996), the *HNRNPK* gene encodes for a 65kDa multi-modular protein that comprises of 463/4 amino acids (Matunis et al., 1992). Four protein isoforms of hnRNP K were first identified with the use of a monoclonal antibody (Dejgaard et al., 1994), but to date, only three alternatively spliced mRNA variants have been described in detail. These three hnRNP K mRNA variants encode for isoform–a and –b with distinct C-terminal ends (Figure 1.2).

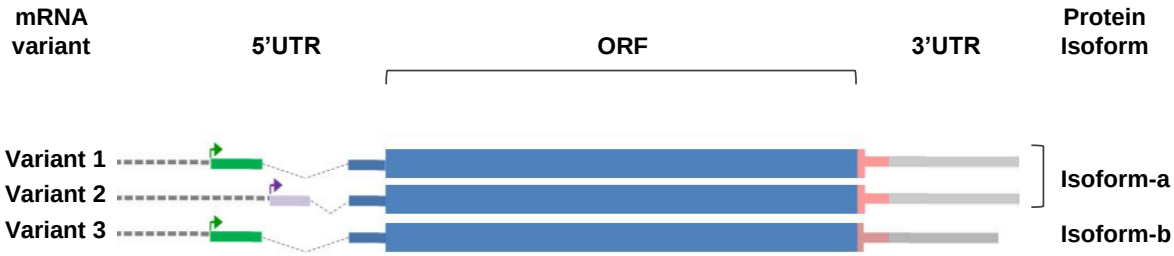


**Figure 1.2 mRNA variants and protein isoforms of hnRNP K**
The three alternatively spliced hnRNP K mRNA variants that have been described in detail are shown. Transcript variants 1 and 3 make use of a different transcriptional start site (green arrow) as compared to variant 2 (purple arrow). Nonetheless, variants 1 and 2 encode for the same protein isoform, isoform–a, while transcript variant 3 uses an alternate acceptor splice site at the last coding exon, resulting in a frame-shift and encodes for isoform–b with a distinct C-terminus. 3'UTR, 3' untranslated region; 5'UTR, 5' untranslated region; ORF, open reading frame.

In addition to the three KH domains and NLS that all PCBPs possess, hnRNP K also contains a hnRNP K interactive (KI) as well as a nuclear shuttling (KNS) domain. The KH domains mediate hnRNP K's binding to DNA and RNA, where the full cooperation between KH domains is required for a three-pronged interaction with DNA/RNA in order to create binding of high affinity and specificity (Baber et al., 2000; Dejgaard and Leffers, 1996; Siomi et al., 1994). In addition, hnRNP K has been shown to preferentially bind single-stranded DNA over either RNA or native double-stranded DNA (Tomonaga and Levens, 1995).

The bipartite-type NLS and KNS domain on hnRNP K allows for its shuttling between the nucleus and cytoplasm to perform its diverse functions (Michael et al., 1997), while the KI domain is responsible for its interaction with many other proteins (Bomsztyk et al., 2004). As shown in Figure 1.3, the KI region contains proline rich docking sites (Arg-X-X-Pro-X-X-Pro and Pro-X-X-Pro-X-Arg, where X is any amino acid) that can interact with Src-homology-3 (SH3) domains containing kinases such

as Src, Lck, Fyn, Vav and Lyn (Van Seuningen et al., 1995). Interactions with other proteins involved in gene expression, such as TATA box binding protein (TBP) and Zik1, are also mediated via the KI domain, though the structural basis for such interactions is not clear (Bomsztyk et al., 2004).

Genetic studies on hnRNP K protein orthologs found in other species revealed the importance of hnRNP K protein. hnRNP K protein is highly conserved within mammals while the three KH domains of the mammalian hnRNP K are almost completely conserved in *Xenopus laevis* and well-conserved in *Caenorhabditis elegans*, *Drosophila melanogaster* as well as *Saccharomyces cerevisiae* (Bomsztyk et al., 2004; Siomi et al., 1993). However, the group of proline-rich SH3-docking sites located at the KI domain found in mammalian and *X. laevis* hnRNP K proteins are absent in other orthologs. Additionally, the KNS domain is only found in mammalian hnRNP K. These findings suggested that the KI and KNS domains only emerged at a later stage in evolution, allowing hnRNP K to gain additional new functions (Bomsztyk et al., 2004).

The multifaceted and complex biological function of hnRNP K

The observation that a large fraction of hnRNP K is not associated with the hnRNP particle has suggested that hnRNP K has many other cellular functions apart from RNA processing (Bomsztyk et al., 1997). Indeed, in addition to the various different structural domains that it possesses, hnRNP K can be subjected to a variety of post-translational modifications such as ubiquitination, phosphorylation by different kinases such as Src, JNK, ERK and PKCδ (Habelhah et al., 2001a; Habelhah et al., 2001b; Ostareck-Lederer et al., 2002; Ostrowski et al., 2000; Schullery et al., 1999) or methylation by PRMT1 (Figure 1.3) (Chan et al., 2009; Chiou et al., 2007).

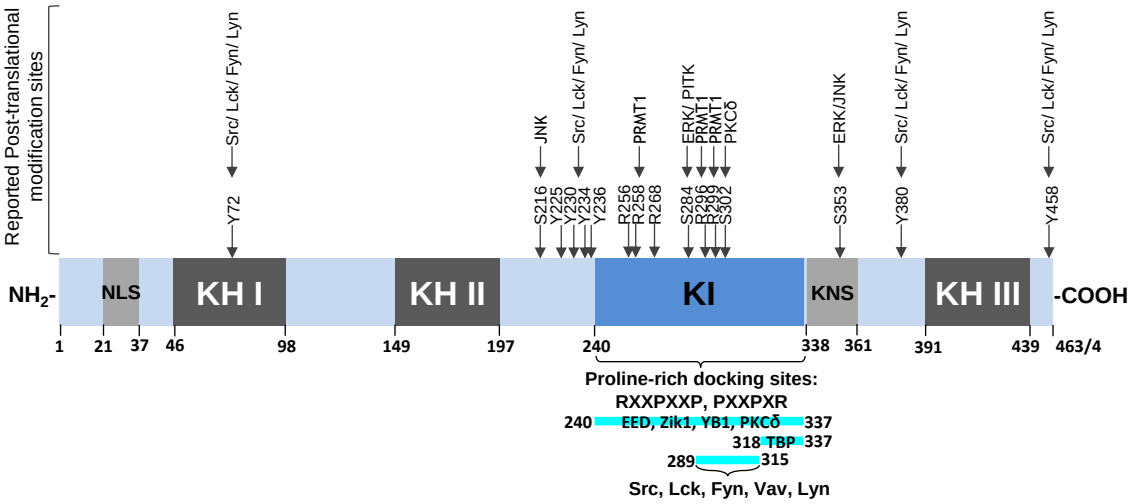


**Figure 1.3 Modular structure of the multifunctional hnRNP K**

hnRNP K contains three KH (hnRNP K homology) domains responsible for its binding to nucleic acids, as well as the NLS (nuclear localisation signal) and KNS (hnRNP K nuclear shuttling) signal that allows it to shuttle between the nucleus and cytoplasm. It possesses a KI (hnRNP K interactive) domain that contains proline-rich docking sites that allows for interaction with multiple proteins and kinases (interaction regions highlighted in cyan) such as TBP, Src and Lck. Multiple amino acid residues on hnRNP K could be post-translationally modified by various kinases e.g. Src, JNK as well as arginine methylases e.g.PRMT1 that have been experimentally verified, as indicated by the black arrows. These have together contributed to the multifaceted and complex biological functions of hnRNP K. Numbers of amino acid residues indicate the respective human sequence. R, Arginine; P, Proline; X, any amino acid.

There are up to 73 potential phosphorylation sites, including tyrosine residues that can serve as a docking sites for proteins with Src-homology-2 (SH2) domains upon phosphorylation (Mikula et al., 2006). As such, hnRNP K can mitigate extracellular changes when the various phosphorylation sites are modified in response to a host of signals. Thus, it has been proposed to be a docking platform that integrates and connects the signals from multiple cascades at sites of nucleic-acid directed processes (Bomsztyk et al., 2004). Furthermore, large scale screening study has identified more

than 140 potential protein binding partners of hnRNP K (Mikula et al., 2006). Hence, taking into account the various domains that hnRNP K possesses, its huge plethora of protein binding partners as well as the multiple amino acid residues that can be post-translationally modified, it is not surprising that hnRNP K has many diverse functions.

Hence, in addition to its involvement in mRNA splicing of genes such as β-tropomyosin gene, glucose-6-phosphate dehydrogenase, Bcl-x and human immunodeficiency virus 1 (Expert-Bezancon et al., 2002; Griffith et al., 2006; Marchand et al., 2011; Revil et al., 2009), hnRNP K has been reported to be a multifunctional protein involved in signal transduction and other aspects of gene expression, e.g. chromatin remodelling, transcriptional regulation, stabilisation of mRNA, as well as translational regulation of genes (Bomsztyk et al., 2004). Of these functions, its roles in transcriptional and translational regulation have been reported fairly extensively.

It has been shown that hnRNP K transcriptionally regulate genes in a manner that requires the CT-element found on the genes' promoter. hnRNP K can associate with TATA box-binding protein (TBP) (Tomonaga and Levens, 1995) to enhance the expression of c-Myc (Michelotti et al., 1996) as well as translation initiation factor eIF4E (Lynch et al., 2005). Together with Sp1, hnRNP K can also transcriptionally activate c-Src via CT-rich regions found on the c-Src promoter (Ritchie et al., 2003). Apart from the transcriptional activation of genes, hnRNP K can also transcriptionally repress genes. hnRNP K was demonstrated to repress the expression of thymidine kinase via the CT motif (Hsieh et al., 1998), as well as the Sp1- and Sp3-mediated transactivation of neuronal acetylcholine receptor β4 subunit via a CT-rich region (Du et al., 1998). Other cofactors associated with hnRNP K also play a part in transcriptional regulation of genes, e.g. the androgen receptor (AR) gene is trans-

repressed by hnRNP K together with Purα (Wang et al., 2008) while the association of hnRNP K, PARP-1, B-myb or c-Myc collectively activates the expression of AR (Shi et al., 2008a). In addition, hnRNP K can also transcriptionally regulate genes in the absence of CT-rich region(s) on the gene's promoter (Lee et al., 1996). For example, hnRNP K can transcriptionally repress COX-2 at NF-κB binding regions (Shanmugam et al., 2008), associate with transcriptional repressors such as Zik1 (Denisenko et al., 1996) and bind to C/EBP-β to repress its transcriptional activity (Miau et al., 1998).

As hnRNP K has been observed to bind translation elongation factor-1α (EF-1α), Bomsztyk et al. (1997) suggested its involvement in translation regulation. Together with PCBP1/2, hnRNP K stimulates an internal ribosome entry site's activity of c-Myc mRNA, thereby translationally activating c-Myc gene expression (Evans et al., 2003). In addition, hnRNP K has been shown to be involved in Angiotensin II stimulation of vascular endothelial growth factor (VEGF) mRNA translation (Sataranatarajan et al., 2008). Apart from translationally activating downstream targets, hnRNP K has been shown to inhibit the translation of AR (Mukhopadhyay et al., 2009) and together with PCBP1/2, repress the translation of human papillomavirus type 16 L2 mRNA (Collier et al., 1998). Moreover, the post-translational modification of hnRNP K, i.e. phosphorylation by different kinases, can greatly affect the translational regulation outcome of genes downstream of hnRNP K, such as the regulation of 15-lipoxygenase (15-LOX) mRNA translation.

Taken together, it has been shown that the biological function of hnRNP K is multifaceted and complex, hence making it an important protein for investigation.

### 1.1.3 Expression of hnRNP K and its association with cancer

Expression and role of hnRNP K in normal cells and development

Given that hnRNP K possesses diverse functions and is involved in a number of fundamental biological processes, it is intriguing to observe that hnRNP K is not ubiquitously expressed. In normal proliferating keratinocytes, only half of them expressed hnRNP K in the G1 and S phase of the cell cycle (Dejgaard et al., 1994). In addition, hnRNP K was found abundantly in most of the major mouse tissues types at varying levels, though it was barely detectable in some. In support of this, Blanchette et al. (2006) reported the differential expression of hnRNP K at different stages of rat embryonic development, as well as the absence of its expression in certain tissues at various stages. hnRNP K was also found to be localised primarily in the nuclei, excluding the nucleoli, with stronger nuclear than cytoplasmic staining (Kamma et al., 1995). Together, these studies have indicated that hnRNP K is likely to function and be regulated in a spatial and temporal manner.

Nevertheless, even though there is no hnRNP K knockout mouse to date, studies on hnRNP K orthologs have revealed its role in normal development. In yeast, hnRNP K orthologs are not essential for growth (Denisenko and Bomsztyk, 2002), but embryonic lethality with a penetrance of 10-25% was observed in *C. elegans* when the expression of hnRNP K ortholog was depleted (Piano et al., 2002). Additionally, mutants of hnRNP K ortholog in *Drosophila* resulted in a wide range of phenotypes, i.e. from normal viability with no defects to very low viability with developmental defects. Thus, hnRNP K was suggested to affect cell proliferation and regulate cell fate (Charroux et al., 1999). Interestingly, the knockdown of hnRNP K ortholog in two-cell stage embryos in *X. laevis* that is the most similar to the mammalian hnRNP K, inhibited axonal outgrowth but did not affect cellular proliferation. This strongly

suggested that hnRNP K is not obligatory in cellular proliferation and that it possesses more diverse functions later in evolution (Liu et al., 2008).

Expression and role of hnRNP K in transformed cells and cancer

Unlike normal cells where hnRNP K is not ubiquitously expressed, increased hnRNP K expression levels have been detected in transformed cells. In contrast to normal proliferating keratinocytes, it was observed that hnRNP K is constitutively expressed throughout cell cycle in simian virus 40-transformed keratinocytes as well as other mammalian cells that were transformed *in vitro* (Dejgaard et al., 1994). Moreover, hnRNP K has been found to be consistently upregulated in many different human cancers (Chen et al., 2008a; Hatakeyama et al., 2006; Klimek-Tomczak et al., 2006; Li et al., 2004; Mandal et al., 2001; Nagano et al., 2004; Perrotti and Neviani, 2007; Pino et al., 2003; Wen et al., 2010; Zhou et al., 2010).

Though the role of hnRNP K in cancer is not well understood, various cellular functions of hnRNP K have suggested its involvement in tumourigenesis. For example, hnRNP K has been demonstrated to positively regulate tumour suppressors such as BRCA1 (Thakur et al., 2003), and serves as a transcriptional cofactor for p53 to bring about DNA-damage-induced cell-cycle checkpoint arrest (Chen et al., 2008b; Moumen et al., 2005). This suggested that hnRNP K can suppress tumourigenesis.

Nonetheless, hnRNP K has also been reported to be regulated by many oncogenic kinases such as Src and ERK (Bomsztyk et al., 2004). In addition, the promoter region of hnRNP K was found to possess upstream binding sites for oncogenes such as E2F, AP1 and c-Myc, indicating that it may be regulated by these oncogenes (Carpenter et al., 2006). Thus, as hnRNP K positively regulates several oncogenes such as c-Myc, c-Src and eIF-4E, this likely forms a positive feedback mechanism to drive

proliferation (Michelotti et al., 1996; Ritchie et al., 2003; Takimoto et al., 1993). This is further supported by the observation that the induction of cell proliferation is associated with increased hnRNP K gene expression (Ostrowski and Bomsztyk, 2003). Moreover, given that enhanced levels of cytoplasmic hnRNP K have been implicated in tumour metastasis (Inoue et al., 2007) and that hnRNP K has also been demonstrated to bind to the telomeric C-rich strand *in vitro*, this suggested its possible involvement in telomere functioning that is important in cellular transformation (Bandiera et al., 2003; Lacroix et al., 2000). Hence, these studies have indicated the potential ability of hnRNP K to drive tumourigenesis.

Intriguingly, the expression levels of hnRNP K were observed to be affected in cells treated with agents associated with cancer growth or therapy. Apart from serum treatment that can increase the levels of hnRNP K, growth factors such as epidermal growth factor and heregulin-β1 (Mandal et al., 2001; Ostrowski and Bomsztyk, 2003) as well as DNA damage inducing agents such as ionizing radiation, ultraviolet treatment and phleomycin were also able to stabilise hnRNP K protein levels (Moumen et al., 2005).

In contrast, a decrease in hnRNP K protein levels was observed in cells treated with anti-cancer drugs such as bicalutamide, mitomycin C, RITA and docetaxel (Barboro et al., 2009; Benelli et al., 2009; Enge et al., 2009; Rahman-Roblick et al., 2007). A camptothecin analogue has also been reported to activate PKCδ, which resulted in the subsequent decrease in hnRNP K protein level (Gao et al., 2009). Apart from being downregulated by activated PKCδ, hnRNP K has been reported to be substrates of various endogenous proteases associated with cell death, including calpain, caspase and all human granzymes (Dix et al., 2008; Kimura et al., 2003; Mahrus et al., 2008; van Domselaar et al., 2012). Thus, the findings from the various

studies have suggested that the hnRNP K levels in the cell have an association with cell survival.

Notably, recent reports have revealed the association of hnRNP K with apoptosis, albeit with contrasting results (Chen et al., 2010; Chen et al., 2009; Gao et al., 2009; Moumen et al., 2005; van Domselaar et al., 2012; White et al., 2010). Taken together, these studies further substantiated the complex role of hnRNP K in tumourigenesis and cancer cell survival, which deserves further characterization.

## 1.2 Apoptosis

### 1.2.1 Physiological and Pathological Significance of Apoptosis

Cell death can be classified via its morphological appearances, enzymological criteria, functional aspects or immunological characteristics. Thus, typical cell death modes can be classified into: apoptosis, autophagy, cornification and necrosis (Kroemer et al., 2009). Apoptosis is one of the programmed cell suicidal mechanism that has been studied in great detail since its first description in 1972 (Kerr et al., 1972). It is an energy-dependent mode of cell death, with classical morphological features, e.g. rounding-up of the cell, reduction of cellular and nuclear volume, nuclear condensation and fragmentation, and plasma blebbing (Kroemer et al., 2009). Since 1972, a large number of genes that control the initiation, execution and regulation of apoptosis in various species have been identified and evidence revealed that the mechanism of apoptosis is evolutionarily conserved (Danial and Korsmeyer, 2004).

In metazoan organisms, apoptosis is a vital cellular process during embryonic development and morphogenesis, normal tissue homeostasis via elimination of unnecessary and defective cells, proper development and functioning of the immune

system, aging, as well as a defensive mechanism against viral infection and the emergence of cancer (Elmore, 2007).

Dysregulated apoptosis, either inadequate or in excess, can therefore result in many undesirable conditions including developmental defects, autoimmune diseases, neurodegeneration, or even cancer. When there is excessive apoptosis, conditions and pathologies can occur, such as excessive scarring and fibrosis during wound healing (Greenhalgh, 1998), autoimmune deficiency syndrome that results from infection with human immunodeficiency virus (Li et al., 1995), neurodegenerative disease such as Alzheimer's disease (Ethell and Buhler, 2003) and Amyotrophic Lateral Sclerosis, as well as ischemia-associated injury such as myocardial ischemia (Freude et al., 2000). On the other hand, inadequate apoptosis results in autoimmune disease such as autoimmune lymphoproliferative syndrome (ALPS) with insufficient death of auto-aggressive T lymphocytes and B lymphocytes (Worth et al., 2006), as well as the formation of many different types of cancer. Thus, apoptosis is an important process that has to be tightly regulated.

### 1.2.2 Caspases – Regulators and Executors of Apoptosis

Caspases have been considered as the central components of apoptotic response (Thornberry and Lazebnik, 1998), belonging to a family of cysteine proteases that typically cleave after an aspartate in their substrates (Alnemri et al., 1996). Even though the first caspase was identified in humans (Thornberry et al., 1992), the critical role that caspases play in apoptosis has only been revealed in *C. elegans* (Yuan et al., 1993). Since then, at least 14 different mammalian caspases have been identified, with 11 of them being found in human (Riedl and Shi, 2004).

Of the 14 different known mammalian caspases, at least seven have been shown to possess important roles in apoptosis (Shi, 2002). These caspases can be generally divided into two groups, namely the initiator caspases, i.e. caspase-2, -8, -9 and-10 and the effector caspases, i.e. caspase-3, -6, -7 (Riedl and Shi, 2004). Initiator caspases are characterized by their N-terminal regions that comprise one or more adaptor domains important for their function, while effector caspases usually contain 20-30 residues in their prodomain sequences. All of the caspases are produced in cells as catalytically inactive zymogens that require proteolytic activation during apoptosis (Riedl and Shi, 2004).

Activation of an effector caspase such as caspase-3 is performed by an initiator caspase such as caspase-8 or caspase-9 via cleavage at specific aspartate residues that separate the large (~p20) and small (~p10) subunits and two of each subunits closely associate with each other to form an active caspase heterotetramer. In contrast, the initiator caspases are auto-activated via the formation of a multi-component complex under apoptotic conditions, which recruits and activates the protease by proximity-induced dimerization. Once the caspase cascade is activated, the effector caspases (caspase-3, -6, -7) are responsible for the cleavage of a broad spectrum of cellular targets that leads to the apoptotic demise of the cell (Riedl and Shi, 2004). Hence, caspases could function as a substrate of another caspase. It should be noted that caspase-8 and -10 are the only caspases that have been demonstrated to cleave a wide array of other caspases including: caspase-1, -2, -3, -4, -6, -7, -8, -9 and -10 (Guo et al., 2002; Srinivasula et al., 1996). Additionally, caspase-3 has been demonstrated to cleave both caspase-8 as well as caspase-9, and is able to cleave caspase-2 and -6 more efficiently than caspase-7 (Sohn et al., 2005; Walsh et al., 2008).

### 1.2.3 Intrinsic and Extrinsic Apoptotic Pathways

The apoptotic response in mammalian cells is mediated mainly via two pathways, namely the intrinsic and the extrinsic pathways, depending on the origin of the death stimuli (Figure 1.4). Apart from these two main pathways, another oligomerisation platform called the PIDDosome has been described in recent years, which can promote the activation of caspase-2 in response to DNA damage via PIDD and RAIDD adaptor proteins. Nonetheless, alternative caspase-2 activation mechanisms may also exist (Manzl et al., 2009).

Intrinsic pathway

The intrinsic pathway is mediated via mitochondria, where cytochrome-C is released into the cytosol when cell stressors such as DNA damage alter the intracellular balance of interactions between pro- and anti-apoptotic members of the Bcl-2 protein family. The released cytochrome-C promotes the assembly of an oligomeric apoptosome that comprises of adaptor Apaf-1 and its cofactor dATP, which then recruits and activates caspase-9, thereby triggering a cascade of caspase activation to bring about apoptosis (Li et al., 1997). Apart from cytochrome-C, the other mitochondrial factors released include: second mitochondrial-derived activator of caspases (SMAC)/ direct inhibitor of apoptosis (IAP)-binding protein with low PI (DIABLO), apoptosis-inducing factor (AIF), endonuclease G (EndoG) and high-temperature-requirement protein A2 (HTRA2/OMI) (Riedl and Salvesen, 2007).

Extrinsic pathway

The extrinsic pathway is initiated by the binding of an extracellular death ligand belonging to the tumour necrosis factor (TNF) superfamily to the death receptors, where activation of initiator caspase-8 and caspase-10 occurs via an oligomerisation platform known as the death-inducing signalling complex (DISC) (Wilson et al., 2009). The death ligands (i.e. FAS-ligand, TRAIL, TNFα) upon binding induce clustering of death receptors such as Fas, DR4 or DR5 at the cell surface that assembles a DISC by further recruiting caspase-8 or -10 via the adaptor Fas-associated death domain (FADD). Dimerization of caspase-8 or -10 within the DISC triggers activation of these initiator caspases, which is reinforced by the excision of the intercatalytic domain linker and by polyubiquitination on the p10 subunit (Fuentes-Prior and Salvesen, 2004; Jin et al., 2009). Apart from the classical DISC described, a cytoplasmic complex nucleated downstream of TNF receptor 1 by kinase RIP-1 and FADD can also activate caspase-8 (Salvesen and Ashkenazi, 2011).

Following the activation of caspase-8 or -10, these initiator caspases can cleave and activate effector caspases directly in Type I cells, or indirectly in Type II cells (Barnhart et al., 2003). In Type I cells, induction of apoptosis by Fas-ligand is executed by activation of large amounts of caspase-8 at the DISC formed, which are sufficient to directly cleave and activate effector caspase-3 to bring about apoptosis (Scaffidi et al., 1999b). However, in the more commonly found Type II cells, such as Jurkat T-lymphocyte cell line and hepatocytes, induction of apoptosis by death ligands produces very little activation of caspase-8 at the DISC and hence requires the amplification of death signals via the intrinsic pathway (Scaffidi et al., 1999b). Caspase-8 would cleave Bid to produce truncated-Bid (tBid) that translocates to the

mitochondria where it induces the release of mitochondrial factors, e.g. cytochrome C, which ultimately enhances the apoptotic signal (Li et al., 1998).

Nonetheless, recent reports have revealed that the distinction between Type I and Type II cells could be a result of the varying levels of cellular factors present. When the levels of these cellular factors are perturbed, activated caspase-8 could result in the direct activation of caspase-3, without the obligatory involvement of mitochondrial signalling as previously observed in Type II. The use of membrane bound Fas-ligand instead of soluble Fas-ligand to induce apoptosis (Schneider et al., 1998), increased cell surface expression levels of the death receptors (Meng et al., 2011), reduction in levels of X-linked IAP (XIAP) (Jost et al., 2009), increased cell adhesion and reduced levels of keratin 8/18-intermediate filament (Gilbert et al., 2008; Walter et al., 2008) could all switch Type II apoptotic signalling to Type I.

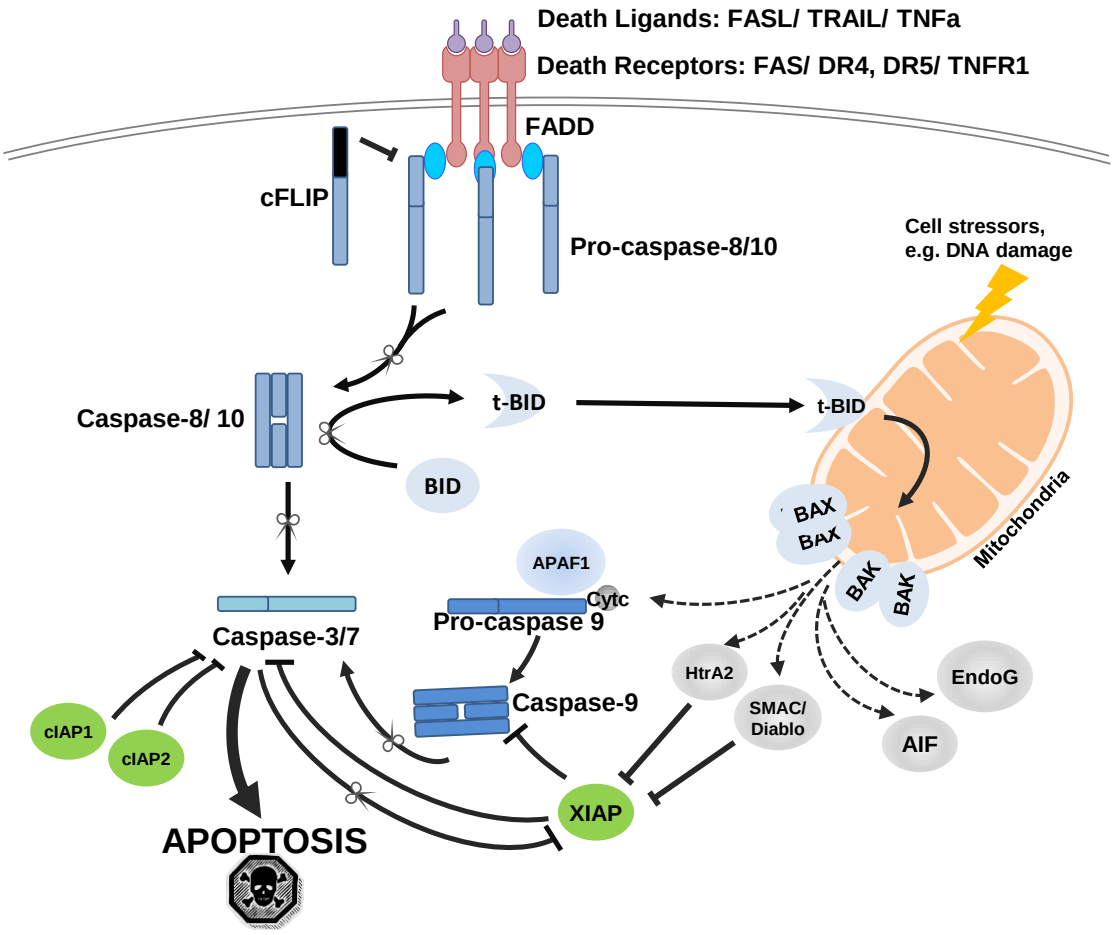


**Figure 1.4 The extrinsic and intrinsic pathways of caspase activation and apoptosis**

The extrinsic pathway involves oligomerisation of death receptors by their ligands, resulting in the formation of DISC that recruits and activates initiator caspase-8/10 that executes apoptosis by cleaving and activating effector caspase-3/7 or by cleaving Bid that translocates to the mitochondria for initiation of the intrinsic pathway. The intrinsic pathway is activated by cell stressors such as DNA damage that alters the intracellular balance of interaction between members of the Bcl2 family, thereby activating the pro-apoptotic Bcl2 family of proteins (green) that triggers mitochondrial release of cytochrome-C into the cytosol. This is necessary for the formation of apoptosome with Apaf-1 and pro-caspase-9 for the activation of caspase-9 and the subsequent activation of caspase-3/7. Caspase inhibitors such as cFLIP, cIAP1, cIAP2 and XIAP are also indicated.

### 1.2.4 Inhibitors of Caspases and Apoptosis

As caspases are the key molecules of apoptosis, moderation of caspase activity is considered to be an important means of regulating apoptosis. Nonetheless, other molecules have also been reported to be able to regulate apoptosis signalling without directly regulating caspases, such as heat shock proteins (Takayama et al., 2003) and Tumour necrosis factor alpha-induced protein 3 (Liuwantara et al., 2006). Various methods of caspase regulation have been described and these include decoy inhibitors, caspase inhibition and caspase degradation (Pop and Salvesen, 2009). Various molecules involved in caspase regulation have also been described, including cellular FLICE-like inhibitory protein (cFLIP) as well as members of the inhibitor of apoptosis (IAP) protein family (Pop and Salvesen, 2009).

Cellular FLICE-like inhibitory protein (cFLIP)

cFLIP was first identified to be a major inhibitor of caspase-8 activation at DISC mediated by death receptors (Irmler et al., 1997). It has several spliced variants and three of them have been characterized: $cFLIP_L$, $cFLIP_S$ and $cFLIP_R$. $cFLIP_S$ and $cFLIP_R$ are shorter isoforms with similar N-terminals but different C-terminals as compared to $cFLIP_L$ (Lavrik and Krammer, 2012). $cFLIP_L$ is structurally similar to caspase-8 with two death effector domains (DEDs) at the N-terminus and a C-terminal caspase-like domain, but its C-terminus lacks the catalytic cysteine residue that confers the proteolytic activity of caspase-8 (Irmler et al., 1997). Nonetheless, all three isoforms of cFLIP are able to bind to DISC and block activation of caspase-8 and apoptosis (Lavrik and Krammer, 2012). However, c-$FLIP_L$ can only inhibit apoptosis when it is present in high concentrations at DISC (Chang et al., 2002; Krueger et al., 2001). When $cFLIP_L$ is present in low concentrations with strong

receptor stimulation or in the presence of one of the short cFLIP isoforms, it can facilitate the activation of caspase-8 at the DISC (Chang et al., 2002; Fricker et al., 2010). cFLIP can be cleaved by pro-caspase-8 at Asp376 to form p43-FLIP and at Asp196 to form p22-FLIP, both of which can induce NF-κB survival signalling (Golks et al., 2006; Scaffidi et al., 1999a).

As cFLIP was detected to be over-expressed in many different cancers and has been associated with increased resistance to Fas- and TRAIL-mediated apoptosis (Korkolopoulou et al., 2004; Okano et al., 2003; Salon et al., 2006; Shirley and Micheau, 2010; Zong et al., 2009), the regulation of c-FLIP was extensively studied. It has been shown to be transcriptionally activated by a wide variety of transcription factors, i.e. NF-κB, p53, hnRNP K, and is transcriptionally repressed by various transcription factors, i.e. c-Myc and c-Fos (Shirley and Micheau, 2010). This accounts for the differential regulation of cFLIP isoforms in a cell-type dependent manner and ultimately determines cell fate from a given stimulus. However, more studies would be required to understand the molecular mechanisms behind the regulation of cFLIP mRNA variants' alternative splicing, which is still poorly understood (Shirley and Micheau, 2010). cFLIP isoforms are also tightly regulated at the post-transcriptional level by a myriad of compounds that can induce the degradation of cFLIP, including chemotherapeutic drugs as well as cellular E3 ubiquitin ligase Itch that is under the control of JNK (Shirley and Micheau, 2010). Hence, coupled with the fact that the isoforms of cFLIP are short-lived proteins (Fulda et al., 2000; Hernandez et al., 2001), degradation of cFLIP protein provides sensitization to apoptosis mediated by death receptors.

Inhibitors of Apoptosis (IAP)

The most well-known inhibitors of caspases belong to the conserved inhibitor of apoptosis (IAP) protein family. The IAPs were originally identified in the genome of baculovirus by their ability to suppress apoptosis in infected host cells (Crook et al., 1993) and have been identified in mammals, *Drosophila*, *C. elegans* and yeast (Wei et al., 2008). There are eight mammalian IAPs, which are XIAP, cIAP1, cIAP2, melanoma IAP (ML-IAP)/Livin, IAP-like protein-2 (ILP2), neuronal apoptosis-inhibitory protein (NAIP), Bruce/Apollon and Survivin (Riedl and Shi, 2004). The defining characteristics of an IAP molecule is the presence of the BIR domain, which can be present in a single copy or up to two to three repeats in the N-terminal portion of IAPs. In addition, the IAPs also contain other functional regions such as a 'Really Interesting New Gene' (RING) domain or caspase-associated recruitment domain (CARD) near the C-terminus (Srinivasula and Ashwell, 2008). IAPs have been demonstrated to inhibit caspase activity by binding directly to caspase at the active site or allosterically and could also be involved in the proteasomal degradation of active caspases or reduce the activity of caspase via the ubiquitin ligase activity that they possess (Wei et al., 2008).

Even though initial biochemical studies using GST-IAP fusion proteins and overexpression analyses demonstrated that human Survivin, XIAP, cIAP1 and cIAP2 can bind and effectively inhibit caspase-3, -7 and-9, subsequent quantitative studies revealed XIAP to be the only bona fide caspase inhibitor (Eckelman and Salvesen, 2006; Eckelman et al., 2006; Srinivasula and Ashwell, 2008).

XIAP is the best characterized IAP thus far, and is recognised as the most potent endogenous caspase inhibitor. XIAP contains three BIR domains, an ubiquitin (Ub)-binding domain and a RING domain. The second BIR domain (BIR2) and linker

region between BIR1 and BIR2 of XIAP inhibits the activity of processed caspase-3 and -7 by binding to their active sites, while its BIR3 domain allosterically inhibits processed caspase-9, sequestering it in a monomeric state (Shiozaki and Shi, 2004). Similar to cIAP1 and cIAP2 that contain the RING domain, XIAP possesses ubiquitin protein ligase (E3) activity and all three of them are able catalyse their own RING-dependent ubiquitination in cells subjected to a pro-apoptotic stimulus (Li et al., 2002; Yang et al., 2000). In addition, it has been demonstrated that XIAP was able to target activated caspase-3 for ubiquitination and proteasomal degradation (Suzuki et al., 2001b), hence downregulating caspase-3 activity (Schile et al., 2008).

Given that IAPs are potent inhibitors of caspases, especially XIAP, it was of no surprise that they were found to be upregulated in cancers (Berezovskaya et al., 2005; Ferreira et al., 2001; Hofmann et al., 2002; Krajewska et al., 2003; Shi et al., 2008b; Shiraki et al., 2003; Tamm et al., 2000). XIAP has been shown to be positively regulated by NF-κB at the transcriptional level (Campbell et al., 2004; Stehlik et al., 1998) and this applies to cIAP1, cIAP2 and Survivin as well (Dutta et al., 2006). Apart from being regulated at the transcriptional level, XIAP has also been demonstrated to be tightly regulated post-transcriptionally and post-translationally (Holcik, 2003; Holcik et al., 2003; Lewis et al., 2007; Sohn et al., 2006) with a short half-life (McNeish et al., 2005; Rosato et al., 2004).

Apart from regulation at the gene expression level, IAPs can also be counteracted by the presence of endogenous cellular factors, i.e. IAP antagonists, during the initiation of apoptosis. Notably, these IAP antagonists have been extensively studied and utilised in the design of anti-cancer drugs, such as the development of SMAC mimetics (Chen and Huerta, 2009; Sun et al., 2008). IAP antagonists are among the mitochondrial factors released via the activation of the intrinsic apoptotic pathway

and the best characterised include SMAC/DIABLO (Du et al., 2000) and HTRA2/OMI (Suzuki et al., 2001a). They have been shown bind and inhibit IAPs directly such as XIAP, cIAP1 and cIAP2, as well as induce their auto-ubiquitination activity, proteasomal degradation or even direct cleavage (Fu et al., 2003; Verhagen et al., 2002; Wei et al., 2008; Wu et al., 2000; Yang et al., 2003).

## 1.3 Hepatocellular Carcinoma (HCC)

### 1.3.1 Epidemiology and etiology of HCC

Being the fifth most common cancer in the world, hepatocellular carcinoma (HCC) is ranked as the third highest cause of cancer-related death globally (El-Serag and Rudolph, 2007). HCC represents the most common type of liver cancer that also broadly includes intrahepatic bile duct carcinoma (cholangiocarcinoma), hepatoblastoma, bile duct cystadenocarcinoma, haemangiosarcoma and epithelioid haemangioendothelioma (Farazi and DePinho, 2006).

Every year, more than half a million people worldwide are diagnosed with HCC. The incidence of HCC varies geographically, reflecting the regional differences in the prevalence of specific etiological factors and ethnicity (El-Serag, 2011). The most prominent risk factors associated with HCC include chronic hepatitis B (HBV) and C (HCV) viral infection, intake of aflatoxin-B1-contaminated food as well as chronic alcohol consumption. In addition, gender can also influence the risk and outcome of HCC, with more males accounting for HCC cases (Farazi and DePinho, 2006).

It has been estimated that 350 million and 170 million people worldwide harbour chronic HBV and HCV viral infections respectively (Cha and Dematteo, 2005). HBV causes an estimated 320,000 deaths annually, with 30-50% of deaths attributable to HCC that chronic HBV carriers have a 100-fold relative risk of developing as

compared to non-carriers (Llovet et al., 2003). Various mechanisms have been proposed for HBV's contribution towards the development of HCC, which includes genome integration, inflammation and p53 inactivation (Farazi and DePinho, 2006). However, with development of the HBV vaccine and administration in endemic areas, the incidence of HCC associated with HBV infection is likely to be reduced within the next generation of people living in the endemic area such as Southeast Asia and sub-Saharan Africa (Feitelson et al., 2002).

On the contrary, an effective vaccine against HCV has not been developed and thus the incidence of HCV infection has been continuously on the rise in economically developed countries, e.g. Japan and the United States (Thomas and Zhu, 2005). Approximately 20% of the chronic HCV cases develop liver cirrhosis and 2.5% develop HCC (Bowen and Walker, 2005). HCV's role in hepatocarcinogenesis has been generally assumed to be mediated via chronic neo-inflammatory process and viral proteins that could have an effect on the host cells that HCV is replicating in (Severi et al., 2010). Nonetheless, these factors presumably contribute to the development of liver fibrosis or cirrhosis, which alone is an independent risk factor for HCC (Farazi and DePinho, 2006).

The clinical outcome of HCC is poor with an average life expectancy of six months from time of diagnosis (Feitelson et al., 2002) and survival rates of 17.5% and 7.3% for untreated cancer over one and two years respectively (Cabibbo and Craxi, 2010). Currently, the most effective treatment options for HCC are surgical resection and liver transplantation, which are applicable only to early HCC. However, most of the HCC patients are diagnosed at advanced stages, hence very few patients are suitable for operative intervention at the time of presentation (Ryder, 2003). As such, the detailed understanding of the molecular mechanisms associated with HCC and the

characterization of potential new gene targets are essential for improving the diagnosis and treatment for HCC.

### 1.3.2 Multistate process of hepatocarcinogenesis

The development of HCC usually progresses from chronic liver cell injury that arises as a consequence of the various etiological agents. Following liver cell injury, inflammation arises with hepatocyte regeneration and liver matrix remodelling with scar formation in liver fibrosis. Nonetheless, without the resolution of fibrosis, liver cirrhosis occurs that ultimately cumulates in the formation of HCC (Chakraborty et al., 2012).

Hepatocarcinogenesis has been proposed to be a complex multistate process that accumulates random genetic alterations after chronic exposure to several mitogenic and mutagenic environments during liver fibrosis and/or cirrhosis. It involves a normal cell accumulating genetic changes to form the initiated cell, which subsequently undergoes selective clonal expansion to become a pre-neoplastic lesion that develops into a malignant tumour and eventually clinical liver cancer (Wang et al., 2002). In this model described by Wang et al. (2002), with the incorporation of several key dysregulated signalling pathways identified by genomic and gene expression analyses (Feitelson et al., 2002) (Figure 1.5), the molecular pathogenesis of HCC has been described to be accompanied by a sequential loss of differentiation, constitutive activation of selected signalling pathways that resulted in accelerated cell growth, prolonged cell survival and anti-apoptosis. Subsequently, with the eventual loss of normal cell adhesion and extracellular matrix, formation of aggressive and metastatic HCC occurs (Feitelson et al., 2002; Hussain et al., 2007).

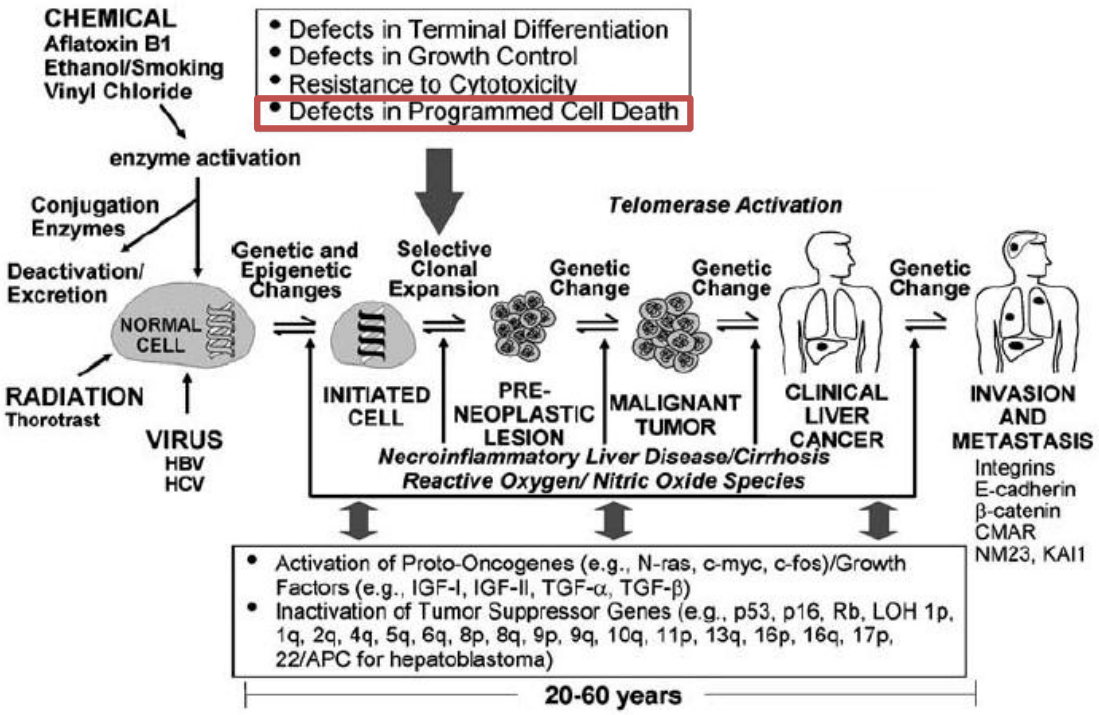


**Figure 1.5 The molecular pathogenesis of HCC**

Hepatocarcinogenesis is a complex, multistep process, where normal hepatocytes accumulate genetic changes over time to form a pre-neoplastic lesion that develops into a malignant tumour and eventually clinical liver cancer. Various signalling pathways are dysregulated during this process, which include increased transforming growth factor-α (TGF-α), insulin like growth factor-2 (IGF-2), Ras-Raf-MAPK as well as NF-κB signal transduction pathways that accelerates hepatocyte growth and increase survival (Benn and Schneider, 1994; Lin et al., 2001; Lucito and Schneider, 1992; Saito et al., 2001). Increased β-catenin in Wnt signalling may also play a role in activating c-Myc and Cyclin D1, and contribute to development of fibrosis and cirrhosis in early HCC as well as aggressive and metastatic HCC (Calvisi et al., 2001). Chromosomal aberrations result in the loss of heterozygosity (LOH) at various chromosomes that causes inactivation of various tumour suppressor genes, which normally limit growth and survival or are involved in other functions such as DNA repair, carcinogen metabolism or protection against oxidative damage. Evasion of apoptosis caused by defects in programmed cell death is also one of the hallmarks of hepatocarcinogenesis (boxed in red) (Feitelson et al., 2002). (Figure adapted from Wang et al. (2002) and reproduced with permission.)

### 1.3.3 Dysregulation of apoptosis in HCC

As in all other cancers, the dysregulation of apoptosis has been listed as a hallmark of HCC, which is deemed a vital contributor to the immunogenic, chemotherapeutic and radiotherapeutic resistance of many human cancers (Fabregat, 2009; Hanahan and Weinberg, 2011).

The dysregulation of apoptosis in HCC comprises of alterations in apoptotic signalling and an increase in survival pathways, such as the disruption of transforming growth factor-beta (TGF-β) signalling (Ito et al., 1991), PI3K/ Akt pathway (Horie et al., 2004) as well as epidermal growth factor (EGF) and Insulin-like growth factor 1 (IGF-1) signalling pathways (Breuhahn et al., 2006; Llovet and Bruix, 2008). Multiple apoptotic signalling pathways are altered, i.e. the extrinsic and intrinsic apoptotic pathways, as well as the disruption of the p53 pathway.

As various chemotherapeutic agents require the activation of p53 to induce apoptosis, the frequent mutation of TP53 gene from arginine 249 to serine associated with the exposure of Aflatoxin B1 is suggested to contribute to the dysregulation of apoptosis in HCC (Bressac et al., 1991; Hsu et al., 1991). However, the prognostic value of p53 in HCC has been controversial, as the overexpression of wild-type p53 has been reported to be associated with a poorer prognosis of patients' survival (Naka et al., 1998), more invasive tumour phenotypes, higher tumour recurrence rates (Jeng et al., 2000) and is likely to play a role in HCC progression, especially at later stages of hepatocarcinogenesis (Ng et al., 1995).

Notably, HCC also shows resistance to apoptosis mediated via the extrinsic apoptotic pathway, where the down-regulation of Fas expression (Lee et al., 2001), FADD and caspase-8 (Shin et al., 1998) have been demonstrated. Nonetheless, it has been suggested that even though the expression of some pro-apoptotic genes

decreased, the dysregulation of the balance between survival and cell death in HCC is mainly due to the overexpression of molecules that counteract apoptosis (Fabregat, 2009). Anti-apoptotic proteins such as cIAP1, Bcl-$x_L$, Mcl1, and Survivin have also been shown to be overexpressed in HCC (Ito et al., 2000; Llovet et al., 2006; Sieghart et al., 2006; Takehara et al., 2001; Zender et al., 2006). Interestingly, XIAP, a caspase-3/7 inhibitor, is expressed at high levels in nearly 90% of clinical tumours from advanced HCC patients and the increased XIAP expression correlates positively with HCC metastasis and resistance to apoptosis (Shi et al., 2008b). In addition, cFLIP, an intracellular inhibitor of caspase-8 activation, has been shown to express constitutively in human HCC cell lines and at higher levels in HCC tissues as compared to non-tumour liver samples (Okano et al., 2003). Thus, the selective inhibition of anti-apoptotic signals in liver tumour cells could serve as a potential therapeutic strategy for the treatment of HCC (Fabregat, 2009).

### 1.3.4 Overexpression of hnRNP K in HCC

As in other cancer cell-types, the overexpression of hnRNP K in HCC has been similarly reported, especially at the protein level. In CBA-T6/W mice that develop spontaneous liver tumours (include hepatocellular adenoma and carcinoma), upregulation of hnRNP K expression at the mRNA and nuclear protein levels was observed in tumour tissues as compared to the surrounding non-tumour liver parenchyma (Ostrowski and Bomsztyk, 2003).

In humans, there were at least five studies that reported the upregulation of hnRNP K in HCC. hnRNP K upregulation was reported in a human HCC cell line, HepG2 (Figure 1.6) as well as in various human HCC samples when compared to normal liver samples in a large scale immunohistochemistry study (Uhlen et al., 2010).

In addition, elevated hnRNP K expression was also reported in proteomic studies on HBV-associated and HCV-associated HCC (Kim et al., 2003; Li et al., 2004; Blanc et al., 2005). Intriguingly, hnRNP K has been associated with both HBV and HCV, which are the important etiological factors for HCC. It has been demonstrated to be a critical host factor that can augment the replication of HBV (Ng et al., 2005; Zhang et al., 2008). In addition, it was revealed to interact with the HCV core protein, which affects the ability of hnRNP K to trans-repress thymidine kinase promoter activity. Hence, Hsieh et al. (1998) proposed that the multiple functions of hnRNP K were disrupted by the core protein of HCV during infection, which could possibly contribute towards HCV pathogenesis.

In a proteomics study that compared differentially expressed proteins in post-liver transplant patients with recurrent and non-recurrent HCC, hnRNP K was once again identified to be upregulated at both the mRNA and protein levels in patients with recurrent HCC (Bai et al., 2009). However, further examination by the authors failed to correlate the levels of hnRNP K in HCC cells studied with their invasiveness; this thus suggested that hnRNP K could play a supporting role in maintaining the growth of aggressive metastatic HCC.

Notably, a recent proteomics study carried out to identify possible biomarkers to distinguish early- and late-stage HCC from liver cirrhosis detected elevated expression levels of nuclear hnRNP K in early- and late-stage HCC samples (Guo et al., 2012). Moreover, the authors also demonstrated that tissue hnRNP K had a higher capability in the detection of early HCC as compared to serum alpha-fetoprotein (AFP).

Taken together, the findings from various studies have suggested a potential role of hnRNP K in hepatocarcinogenesis. Therefore, this warrants a more detailed

characterization of its function in HCC, which may aid in the improvement of this cancer's diagnosis and treatment.

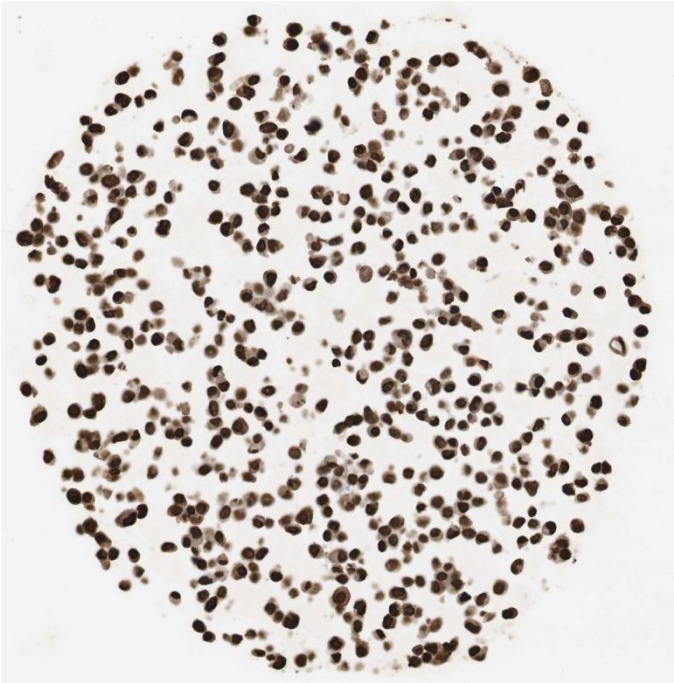

**Figure 1.6 Immunohistochemical staining of HepG2 cells with antibody against hnRNP K**

Immunohistochemical staining of HepG2 cells was done with antibody (sc-28380) from Santa Cruz Biotechnology (brown) and counterstained with methylene blue. Strong nuclear staining revealed high expression levels of hnRNP K in the nucleus of HepG2 cells, with all cells positively stained. (Image was adapted from http://www.proteinatlas.org; Uhlen et al. (2010).)

## 1.4 Aims of Study

hnRNP K is a multifunctional and evolutionally conserved protein involved in a wide variety of biological processes (Bomsztyk et al., 2004), which has been demonstrated to be upregulated in many human cancers including HCC. As HCC is the fifth most common cancer and the third highest cause of cancer-related death globally (El-Serag and Rudolph, 2007), a better understanding of the associated molecular mechanisms and characterization of potential new gene targets would be instrumental for potential improvements in its diagnosis and treatment.

Although the exact role of hnRNP K in cancer is currently not well-understood, its reported elevated levels in various cancer cells coupled with disparate pro- and anti-tumour functions suggested by its cellular functions (described in Section 1.1.3) have highlighted its complex role in tumourigenesis. Intriguingly, hnRNP K levels appear to be associated with cancer cell survival, as exemplified by the its reduction in cells treated with various chemotherapeutic agents (Barboro et al., 2009; Benelli et al., 2009; Enge et al., 2009; Rahman-Roblick et al., 2007). In fact, hnRNP K was recently reported to be downregulated in response to 5-Fluorouracil (5-FU), a chemotherapeutic drug, in colorectal cancer cells and was hence proposed as a predictive marker for 5-FU treatment (Zhang et al., 2010).

Various studies have also suggested that hnRNP K has a role in apoptosis, though with discordant findings (Chen et al., 2010; Chen et al., 2009; Gao et al., 2009; Moumen et al., 2005; van Domselaar et al., 2012; White et al., 2010). Thus, its precise role in apoptosis remains to be elucidated.

Given that apoptosis dysregulation is a hallmark of HCC (Fabregat, 2009), the primary aim of this study is to investigate hnRNP K's role in the dysregulation of apoptosis in human HCC with the use of HCC cell lines. Firstly, the effect of 5-FU on

hnRNP K levels will be examined and verified in HCC cell lines. Consequently, the effect of hnRNP K downregulation on the induction of apoptosis will be investigated, coupled with the elucidation of its underlying mechanism. In addition, hnRNP K's contribution as a transcription factor to the dysregulation of apoptosis in HCC will also be briefly explored. Taken together, the findings from this study would provide a molecular basis for the understanding of hnRNP K's involvement in HCC, which may offer potential improvements in its diagnosis and treatment.

# CHAPTER 2

## Materials and Methods

**Media, Buffers and Solutions**

Media, buffers and solutions utilised in this study are listed in Appendix I.

## 2.1 Cell Culture Techniques

### 2.1.1 Cell lines

The HCC cell lines Hep3B (ATCC-8064) and HepG2 (ATCC HB-8065) were obtained from American Type Culture Collection (Manassas, VA). HCC cell lines HA22T, Huh-1, Huh-4, Tong, PLC/PRF/5, SNU182, SNU449, SNU475, Huh-6, Huh-7, Mahlavu, SK-Hep1, SNU387, SNU398 and SNU423 were kindly provided by WHO Immunology Center, Singapore. The human colorectal carcinoma cell line HCT116 (p53+/+) and its derived isogenic p53-/- cells were kindly provided by Dr. Bert Vogelstein (John Hopkins University, Baltimore, MD).

### 2.1.2 Growth and maintenance of cell lines

The hepatocellular carcinoma and colorectal carcinoma cells stored frozen in liquid nitrogen were thawed quickly in 37°C water bath and re-suspended in 12 ml of Dulbecco's modified Eagle Medium supplemented with 10% Fetal Bovine Serum (DMEM/ 10% FBS) (Appendix I) under sterile conditions. Following centrifugation at 800 rpm for 5 min, pelleted cells were gently re-suspended in 7 ml of DMEM/ 10% FBS and transferred into 25 ml culture flasks (Falcon, BD Biosciences, Franklin Lakes, NJ). The cell cultures were then maintained at 37°C in a humidified incubator with 5% carbon dioxide in atmospheric air. Density of the cells was monitored daily and culture medium was changed whenever necessary. Upon attaining 85-100% confluency, cells were trypsinised in 0.25% trypsin/ EDTA (Invitrogen, Carlsbad, CA) for 5 min at 37°C and reaction was halted with DMEM/ 10% FBS. All of the cells

were then transferred into a 75 ml culture flask and maintained in the 37°C, humidified incubator with 5% carbon dioxide in atmospheric air. Thereafter, depending on the number of cells required for experiments, cells were maintained either in 75 ml or expanded into 150 ml culture flasks.

### 2.1.3 Cryopreservation of cells

Having attained 80-90% confluent growth, cells cultured in 150 ml flask were trypsinised and pelleted down by centrifugation at 1000 rpm for 5 min. After discarding the supernatant, cell pellet was re-suspended in 5 ml of ice-cold freezing medium (Appendix I) and transferred 1 ml per tube into CryoTube vials (Thermo Fisher Scientific, Rochester, NY). These CryoTube vials were stored at -80°C in Cryo 1°C Freezing Container (Thermo Fisher Scientific) filled with 100% isopropanol overnight, before they were transferred to liquid nitrogen for long term storage.

### 2.1.4 Harvesting cell lines

Once the required cell confluency or the specific time-point was attained, cell cultures in the cell culture flasks or plates were trypsinised and pelleted down by centrifugation for 5 min at 1000 rpm. Cell pellets were then washed with 1 ml of phosphate buffered saline (PBS) (Appendix I) each and transferred into 1.7 ml eppendorf tubes. These tubes were then further centrifuged at 500 × *g* for 5 min. Supernatants were discarded from the eppendorf tubes, washed with 500 µl of PBS and centrifuged at 500 × *g* for 5 min again. After the supernatants were discarded, cell pellets were then subjected to total RNA extraction, lysate collection or storage at -80°C.

## 2.2 Polymerase Chain Reaction (PCR)

### 2.2.1 Total RNA extraction

To verify the downregulation of hnRNP K or p53 by siRNA, as well as the verification of downstream genes regulated by hnRNP K, total RNA from Hep 3B and HepG2 transfected with siRNA specific against hnRNP K as well as HepG2 transfected with siRNA specific against p53 was extracted using the RNA extraction kit with DNase-I treatment (NucleoSpin® RNA II, Machery-Nagel GmbH & Co. Kg., Duren, Germany) according to manufacturer's instructions. Newly extracted total RNA was subjected to 65°C for 10 min and was transferred immediately onto ice for 5 min to denature the secondary structure of RNA. RNA concentration was subsequently quantified by Nanodrop (ND-1000) (Nanodrop Technologies, Delaware, USA), and RNA was subjected to cDNA synthesis, followed by either gel-based real-time quantitative Reverse Transcription PCR (RT-PCR) or two-step real-time quantitative RT-PCR.

### 2.2.2 Complementary DNA (cDNA) synthesis

First strand cDNA was synthesised using Maxima® First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to manufacturer's instructions with 1 μg of total RNA as template. Reaction was first incubated for 10 min at 25°C followed by 15 min at 50°C and terminated upon incubation at 85°C for 5 min. First strand cDNA was then diluted 10 times with RNase/DNase free water (Invitrogen), aliquoted and stored at -20°C until next use.

### 2.2.3 Gel-based semi-quantitative RT-PCR

RT-PCR was carried out using ABI GeneAmp PCR 9700 machine, in a reaction volume of 20 µl that contained 2 µl of 10x diluted cDNA as template, 0.5 µM each of the forward and reverse primers (Table 2.1) and 10 µl of the 2x High Fidelity PCR Master Mix (Roche Applied Science, Mannheim, Germany). After denaturing at 94°C for 2 min, amplification was carried out for 25-35 cycles each consisting of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 20-60 sec. Thereafter, a final extension was carried out at 72°C for 7 min before cooling down to 4°C. PCR products were electrophoresed on 1-1.5% agarose gel precasted with GelRed™ (Biotium, Hayward, CA) in 1x TBE buffer (Appendix I). Gels were visualised with UV transilluminator and imaged for recording purposes.

**Table 2.1 Oligonucleotide primers used in gel-based semi-quantitative RT-PCR**

| Gene | Primer Name | Primer Sequence (5'-3') | Product size (bp) |
|---|---|---|---|
| *HNRNP K* | hnRNP K-3-F2 | TGGTCAGCGGATTAAACAAA | 232 (Isoform–a); 172 (Isoform–b) |
| | hnRNP K-3-R | GAAAAACTTTCCAGAATACTGCT | |
| *GAPDH* | GAPDH-F | ACCACAGTCCATGCCATCA | 452 |
| | GAPDH-R | TCCACCACCCTGTTGCTGTA | |

### 2.2.4 Real-time quantitative RT-PCR

Knockdown effect of genes with the use of gene specific siRNAs as well as the gene expression of the potential downstream targets of hnRNP K were examined with two-step real-time quantitative RT-PCR. Following total RNA extraction, first strand cDNA was synthesised as described above. 2 µl of the 10x diluted cDNA was utilised as the template in a 10 µl reaction in triplicates using KAPA SYBR® FAST Roche LightCycler®480 2x qPCR Master Mix (KapaBiosystems, Woburn, MA) according to manufacturer's instructions in the Roche LightCycler® 480 System (Roche Applied Science). Primers designed to span across introns of genes are listed in Table 2.2. Hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) housekeeping gene was used for normalisation.

**Table 2.2 Oligonucleotide primers used in real-time quantitative RT-PCR**

| Gene | Primer Name | Primer Sequence (5'-3') | Product size (bp) |
|---|---|---|---|
| *HNRNP K* | hnRNP K-123-F1 | TTCAGTCCCAGACAGCAGTG | 165 |
| | hnRNP K-123-R1 | TCCACAGCATCAGATTCGAG | |
| *TP53* | p53-F | CCAGGGCAGCTACGGTTTC | 205 |
| | p53-R | CTCCGTCATGTGCTGTGACTG | |
| *TNFSF10* | TRAIL-F | ACCAACGAGCTGAAGCAGAT | 141 |
| | TRAIL-R | ACGGAGTTGCCACTTGACTT | |
| *BIRC2* | BIRC2-F | CCAAGTGGTTTCCAAGGTGT | 145 |
| | BIRC2-R | ATTGGTGGGTCAGCATTTTC | |
| *BIRC3* | BIRC3-F | CCAAGTGGTTTCCAAGGTGT | 119 |
| | BIRC3-R | TGGGCTGTCTGATGTGGATA | |
| *CFLAR* | CFLAR-F | GGACCTTGTGGTTGAGTTGG | 149 |
| | CFLAR-R | AACTTGTCCCTGCTCCTTGA | |
| *XIAP* | XIAP-F | GGGGTTCAGTTTCAAGGACA | 183 |
| | XIAP-R | CGCCTTAGCTGCTCTTCAGT | |
| *HPRT* | HPRT-F | GTAATGACCAGTCAACAGGGGAC | 177 |
| | HPRT-R | CCAGCAAGCTTGCGACCTTGACCA | |

## 2.3 Molecular Cloning

### 2.3.1 Gene ORF cloning

The following section describes the steps undertaken in generating pXJ40-HA-hnRNP K–a and –b (pXJ40-HA-hnRNP K-(a/b)) expression plasmids that expresses hnRNP K isoform–a and –b respectively.

Insert preparation

To prepare the insert DNA, the ORF of hnRNP K (isoform–a or –b) previously amplified from HepG2 and cloned into pcDNA3.1+ (Ng et al., 2005) were utilised as templates for PCR with the use of High Fidelity PCR Master Kit (Roche Applied Science) in ABI GeneAmp PCR 9700 system following the manufacturer's instructions. The primer sequences employed to generate DNA inserts are listed in Table 2.3. The PCR conditions are as outlined: After denaturing at 94°C for 2 min, amplification was carried out for 25 cycles each consisting of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 2 min before a final extension at 72°C for 20 min and cooling down to 4°C. PCR products generated were monitored for specificity and quality by 1-1.5% agarose gel electrophoresis. Subsequently the PCR products were gel-purified with QIAquick Gel Extraction Kit (Qiagen Gmbh, Hilden, Germany) according to manufacturer's instructions.

Enzyme Digestion

The purified PCR products and parental pXJ40-HA plasmid vectors were digested with the *XhoI* and *SmaI* for 2 h at 37°C in a 40-100 µl total reaction volume containing either 1-6 µg of PCR products or 6 µg of vector plasmid, 0.1 total volume of 10x appropriate NEB buffer, 1 µl 100x BSA (10 mg/ml), and 5-20 U of specific

restriction enzyme(s) (New England Biolabs, Ipswich, MA). Specific digested products with sticky ends generated were electrophoresed on 1-1.5% agarose gel and subsequently gel purified with QIAquick Gel Extraction Kit (Qiagen Gmbh, Hilden, Germany) according to manufacturer's instructions.

Ligation

Ligation reactions were performed for 30 min at room temperature in a 20 µl reaction comprising of 4 µl 5x ligation buffer, 1 µl of linearised and gel-purified vector DNA, 1 µl of T4 DNA ligase (Invitrogen) and 1-14 µl of DNA insert(s).

Transformation

Library efficiency® DH5α™ chemically competent *Escherichia coli* (Invitrogen) were employed for transformations. For each transformation reaction, 50 µl of the competent cells was first aliquoted into a 5 ml sterile polypropylene falcon tube, and 1-2 µl of ligated product/ cloning reaction product was added in subsequently, followed by gentle mixing with tapping and 30 min incubation on ice. Mixture was heat shocked at 42°C for 45 sec, followed by immediate incubation on ice for 2 min. 250 µl of S.O.C medium (Invitrogen) at room temperature or preheated to 42°C (for site-directed mutagenesis) was added into each reaction. Recovery of cells was performed, with reactions subsequently incubated at 37°C incubator with shaking at 225 rpm for 40-60 min. 100 µl of the mixture was then plated out on a pre-warmed Luria-Bertani (LB) (Appendix I) plate containing 100 µg/ml ampicillin (Appendix I) and incubated overnight at 37°C to allow colonies of transformants to form.

Screening for Positive Clones

6-8 colonies per plate were chosen and inoculated in 3 ml of LB containing 100 μg/ml ampicillin (Appendix I) with shaking incubation at 37°C overnight. Small-scale preparation of plasmid DNA was then carried out with QIAprep® Miniprep Kit (Qiagen) according to manufacturer's instructions. Isolated plasmids were further verified using enzyme digestion, followed by sequencing analysis.

Sequencing Analysis

Sequencing analyses were done by AITbiotech Pte Ltd, Singapore. Sequencing primers used are summarised in Table 2.4.

**Table 2.3 Oligonucleotide primers used in molecular cloning**

| Type of Cloning* | Product | Primer Name | Primer Sequence (5'-3') |
|---|---|---|---|
| ORF | hnRNP K-isoform-a | PXJ40-hnRNP K-F | AAACTCGAGATGGAAACTGAACAGCCAGAA |
| | | PXJ40-hnRNP K-A-R | AAACCCGGGCTAGAATCCTTCAACATCTG |
| ORF | hnRNP K-isoform-b | PXJ40-hnRNP K-F | AAACTCGAGATGGAAACTGAACAGCCAGAA |
| | | PXJ40-hnRNP K-B-R | AAACCCGGGCTAGAAAAACTTTCCAGAATA |
| SDM | hnRNP K-isoform-a/b-SDM1 | hnRNP K-SDM-1F | CTACACAAGTAACTATACCCAAAGATTTGGCTG |
| | | hnRNP K-SDM-1R | CAGCCAAATCTTTGGGTATAGTTACTTGTGTAG |
| SDM | hnRNP K-isoform-a/b-SDM2 | hnRNP K-SDM-2F | CTATACCCAAGGATTTGGCTGGATCTATTATTG |
| | | hnRNP K-SDM-2R | CCAGCCAAATCCTTGGGTATAGTTACTTGTG |
| SDM | hnRNP K-isoform-a/b-SDM3 | hnRNP K-SDM-3F | CCCAAGGATTTGGCAGGATCTATTATTGGC |
| | | hnRNP K-SDM-3R | CAATAATAGATCCTGCCAAATCCTTGGGTATAG |
| SDM | hnRNP K-isoform-a/b-SDM4 | hnRNP K-SDM-4F | GGATTTGGCAGGATCAATTATTGGCAAAGGTG |
| | | hnRNP K-SDM-4R | CTTTGCCAATAATTGATCCTGCCAAATCCTTG |

*ORF, Open reading frame; SDM, Site-directed mutagenesis.

**Table 2.4 Oligonucleotide primers used for sequencing**

| Primer Name | Primer Sequence (5'-3') |
|---|---|
| T7-Promoter | TAATACGACTCACTATAGGG |
| PXJ40-R | AAGCTGCAATAAACAAGTTCTGCT |

### 2.3.2 Site-Directed Mutagenesis

For the generation of hnRNP K expression plasmids that harbours the four silent mutations, cloning was carried out using QuikChange® Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions with minor modifications. Briefly, pXJ40-HA-hnRNP K-(a/b) expression plasmids generated as outlined in Section 2.3.1 was utilised as the first set of templates in the PCR reactions to generate the first set of expression plasmids harbouring the first silent mutation with primers carrying single nucleotide mutation (Table 2.3). The PCR conditions are outlined as follows: After denaturing at 95°C for 30 sec, amplification was carried out for 14 cycles each consisting of denaturing at 95°C for 30 sec, annealing at 55°C for 1 min and extension at 68°C for 7.5 min before cooling down to 4°C for 2 min.

After which, reactions were incubated at 37°C for 1 h with 10 U of DpnI restriction enzyme provided in the kit, where parental strands without the single nucleotide mutation were digested. Subsequently, 1 µl of the DpnI-treated DNA was added into 50 µl of XL1 Blue Supercompetent cells for transformation and heat shocked. Thereafter, 500 µl of pre-warmed 42°C S.O.C. medium (Invitrogen) was added into each reaction and incubated at 37°C for an hour with shaking at 225 rpm. 100 µl of the mixture was then plated onto LB plate containing 100 µg/ml of

ampicillin. Plasmid purification was then carried out and positive clones were verified by sequencing analysis with primers listed in Table 2.4. Having obtained the correct clones, the expression plasmids were utilised as templates for the next round of site-directed mutagenesis with the subsequent set of site-directed mutagenesis primers (Table 2.3). This was done thrice till the plasmids harbouring the four silent mutations, SDM-pXJ40-HA-hnRNP K (a/b), were obtained.

## 2.4 Transfection

Transfection of expression plasmid(s) and/ or siRNA into the different cell lines was mediated by Lipofectamine2000 reagent (Invitrogen) with Opti-MEM® (Invitrogen). The optimal cell seeding amount for transfection of plasmid DNA and/ or siRNA for different cell lines are described in this section. Following 18-24 h post-transfection, medium was discarded and replenished with fresh DMEM/ 10% FBS.

### 2.4.1 siRNA Transfection

The siRNA oligos utilised in this study were Stealth RNAi$^{TM}$ siRNA synthesised by Invitrogen, Life Technologies Corp. (Carlsbad, CA) as listed in Table 2.5. The oligos were re-constituted in RNase-free water (supplied by Invitrogen) to obtain a stock concentration of 100 μM. Hep 3B and HepG2 were seeded at $0.65 \times 10^6$ cells and $1.2 \times 10^6$ cells per well respectively in 6-well plate (BD, Falcon) and transient transfection of siRNA (10-40 nM) was mediated by Lipofectamine2000 reagent (Invitrogen) according to manufacturer's instruction. In cases where the concentrations of gene-specific siRNA varied, non-specific control siRNA was added to ensure that equal amount of siRNA was transfected for each reaction.

**Table 2.5 siRNA oligos utilised for transfection**

| Gene | Naming of siRNA used in study | Product ID |
|---|---|---|
| *HNRNP K* | si-hnRNP K / hnRNP K siRNA 1 | HSS179311 |
| | hnRNP K siRNA 2 | HSS179312 |
| *TP53* | si-p53 | HSS186390 |
| *CASP8* | si-caspase-8 | HSS141460 |
| *CASP9* | si-caspase-9 | HSS141464 |
| Non-specific control siRNA | si-ctrl | 12935-300 |

### 2.4.2 Plasmid transfection

To examine the anti-apoptotic function of hnRNP K in TRAIL-induced apoptosis, expression plasmids, SDM-pXJ40-HA-hnRNP K-a and SDM–pXJ40-HA-hnRNP K-b were transiently transfected individually into both Hep 3B and HepG2 cells for the overexpression of hnRNP K isoform–a and –b for 30 h before the addition of TRAIL. Briefly, Hep 3B and HepG2 were seeded at $0.65 \times 10^6$ cells and $1.2 \times 10^6$ cells per well respectively in 6-well plate (BD, Falcon) and transient transfection of 2 μg of expression plasmids was mediated by Lipofectamine2000 reagent (Invitrogen) according to manufacturer's instruction.

### 2.4.3 Co-transfection of expression plasmid together with siRNA

To restore the declining levels of hnRNP K for the examination of the specificity of hnRNP K in apoptosis induction, different combinations of (20 nM) siRNA (si-ctrl or si-hnRNP K) and (2 μg) expression plasmids (pXJ40-HA-hnRNP K –a or –b or

SDM–pXJ40-HA-hnRNP K–a or –b) were transiently transfected into both Hep 3B and HepG2 cells. Briefly, Hep 3B and HepG2 were seeded at $0.65 \times 10^6$ cells and $1.2 \times 10^6$ cells per well respectively in 6-well plate (BD, Falcon) and transient co-transfection of 20 nM of siRNA and 2 μg of expression plasmids was mediated by Lipofectamine2000 reagent (Invitrogen) according to manufacturer's instruction.

## 2.5 Western Blot Analysis of Protein

### 2.5.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein Lysate Extraction

For total cell lysate extraction, cultured cell samples were washed twice with PBS, and lysed with NP-40 lysis buffer (Appendix I) containing 1x complete mini protease inhibitor mixture (Roche Applied Science) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO) on ice for 30 min. After centrifuging lysates at 13,200 rpm at 4°C for 15 min, supernatants containing protein were aspirated and measured by Bradford method using a Protein Assay kit (Bio-Rad) to determine their concentration, with reference to four different bovine serum albumin (BSA) standards. All samples were stored in -80°C immediately until use.

Preparation of SDS-PAGE Gels

Resolving gels with acrylamide concentrations of 10% and 12.5% were employed in this study, and stacking gel of 5% acrylamide concentration was consistently applied. Gels were casted using the Mini-PROTEAN electrophoresis cell apparatus (Bio-Rad) according to manufacturer's instructions. Recipes for SDS-

polyacrylamide gels are listed SDS- polyacrylamide gel recipe in Appendix I and the components were added in and mixed in the order shown.

Preparation of Samples and Electrophoresis

Protein samples were mixed with equal amount of 2x SDS-PAGE sample buffer (Appendix I) and heated for 6 min at 95°C. The PageRuler$^{TM}$ Prestained Protein Ladder (Thermo Fisher Scientific) was used as a marker as an indication of the protein molecular weight and for transfer efficiency. Approximately up to a maximum of 35 µl of each sample mixture with sample buffer could be loaded into each well. Electrophoresis was carried out in 1x Tris-Glycine SDS-PAGE running buffer (Appendix I) under a constant current of 20 mA per gel. Electrophoresis was stopped when the dye front reached the bottom of the separating gel and gel was removed from the gel apparatus for subsequent western blot.

### 2.5.2 Western blot

Approximately 20-30 µg of protein was separated on 10% and 12.5% SDS- gel electrophoresis, and transferred onto Immobilon-P$^{SQ}$ PVDF membranes (Millipore, Billerica, MA) with semi-dry Transblot apparatus (Bio-Rad) after gel was equilibrated in 1xTransfer buffer (Appendix I) and PVDF membrane was activated with methanol and equilibrated in 1x Transfer buffer. A sandwich was assembled by placing an extra thick filter paper (100 × 70mm, Bio-Rad) pre-soaked in 1x Transfer buffer onto the platinum anode, followed by the pre-wetted PVDF membrane, the equilibrated gel, and another sheet of soaked filter paper. Air bubbles were carefully removed from in between each layer. Cathode and the safety cover were subsequently placed onto the

stack, and the electrophoretic transfer was performed at a constant voltage of 20 V for 30 min at room temperature.

After blocking with 5% non-fat milk in TBST buffer (Appendix I) overnight at 4°C, antibodies against each protein of interest (as reflected in Table 2.6) were used as primary antibodies for protein detection, followed by reacting to the appropriate HRP-conjugated Goat-anti-Mouse or Donkey-anti-Rabbit secondary antibodies (Dako, Denmark). Equal loading of protein samples was verified with antibodies to β-actin. After extensive washing with TBST buffer, immunoreactive signals on membranes were visualised by reacting with SuperSignal West FemtoChemiluminescent Substrate reagents (Thermo Fisher Scientific) according to manufacturer's instructions, followed by exposure to the Super RX Fuji Medical X-Ray film (Fujifilm Corporation, Tokyo, Japan).

**Table 2.6 Primary antibodies used for western blot analyses**

| Antibody | MW (kDa) | Dilution | Source* | Company | Catalogue number |
|---|---|---|---|---|---|
| Anti-hnRNP K (6466) | 64 | 1:1000 | Rb | iDNA | (custom) |
| Anti-β-actin | 42 | 1:100,000 | Mu | Millipore | MAB1501 |
| Anti-p53 (DO-1) | 53 | 1:3000 | Mu | Santa Cruz Biotechnology | sc-126 |
| Anti-cleaved PARP1 | 89 | 1:1000 | Rb | Cell Signaling | #9541 |
| Anti-HA | - | 1:1000 | Rb | Sigma-Aldrich | H6908 |
| Anti-BID | 22 | 1:1000 | Rb | Cell Signaling | #2002 |
| Anti-caspase-3 | 35,19,17 | 1:1000 | Rb | Cell Signaling | #9662 |
| Anti-caspase-8 | 55 | 1:200 | Mu | Santa Cruz Biotechnology | sc-56070 |
| Anti-XIAP | 55 | 1:200 | Mu | Abcam | ab28151 |

*Mu, Mouse; Rb, Rabbit

## 2.6 Immunofluorescence Staining

Subcellular localisation of hnRNP K was examined with immunofluorescence staining in untreated Hep 3B or Hep 3B cells transiently transfected with si-ctrl or si-hnRNP K. Briefly, Hep 3B cells were seeded at $0.13 \times 10^6$ cells per well into 12-well plates (BD, Falcon) with coverslips with or without transient transfection. 48 h post-transfection or seeding, cells on coverslips were rinsed twice with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) (Appendix I) for 30 min at room temperature. Fixed cells were then rinsed with PBS thrice and permeabilised with 0.1% Triton-X100 (Bio-rad) for 10 min. Cells were subsequently blocked with 1% BSA in PBST (Appendix I) for 1 h at room temperature before 2 h incubation at room temperature with primary antibody Anti-hnRNP K (6466) diluted in 1% BSA in PBST. Cells were washed thrice with PBST before incubation with goat-anti-rabbit secondary antibodies conjugated with Alexa Fluor® 488 (to view with FITC channel) or Alexa Fluor® 546 (to view with RhoD channel) for 1 h at room temperature. Cells were washed thrice with PBST before they were mounted with the Prolong Gold Antifade Reagent with DAPI (Molecular Probes). Cells were observed under the Zeiss Fluorescence Microscope (Carl Zeiss AG, Oberkochen, Germany) with the FITC and DAPI or RhoD and DAPI channels and imaged with the attached Canon Powershot camera (Canon, Tokyo, Japan).

## 2.7 WST-1 Assay

Proliferation of viable cells in monolayer was analysed with the use of a modified MTT assay, WST-1 reagent (Roche Applied Science), according to the manufacturer's instructions. In brief, Hep 3B and HepG2 cells that were seeded at $0.65 \times 10^6$ cells and $1.2 \times 10^6$ cells per well respectively in 6-well plates were

transiently transfected with siRNA. 24 h following transfection, cells were trypsinised with 0.05% trypsin/ EDTA (Invitrogen) and re-seeded into 96-well plates at a concentration of 1.5-2 × $10^4$ cells/well in 100 µl DMEM/10%FBS in triplicates and cultured for another 2-4 days. At each time point, 10 µl of WST-1 reagent was added into each well and further incubated for 40 min at 37°C. The absorbance was measured with Infinite® 200 PRO plate reader (Tecan, Männedorf, Switzerland) at 450 nm with reference at 690 nm.

### 2.8 TUNEL Assay

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was carried out with *in-situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science), according to manufacturer's instructions. Briefly, Hep 3B cells seeded in 6-well plates at 0.65 × $10^6$ cells per well were transiently transfected with siRNA only, or co-transfected with 2 µg of pXJ40-HA or SDM-pXJ40-HA-hnRNP K-(a/b) together with 20 nM of si-ctrl or si-hnRNP K. Following 48 h post-transfection, cells were trypsinised with 0.05% trypsin/ EDTA (Invitrogen), re-seeded into 4-well culture slide (BD Biosciences) at 1 × $10^5$ cells/well in 500 µl DMEM/ 10% FBS and were cultured for additional 48 h. Cells on culture slides were rinsed twice with PBS and fixed with 4% paraformaldehyde for 1 h at room temperature with shaking. Fixed cells were then rinsed with PBS thrice and permeabilised with 1% Triton-X-100 in 0.1% sodium citrate for 2 min on ice. Cells were washed with PBS again before labelling with the TUNEL reaction mixture for 1 h in dark at 37°C. Following labelling, cells were rinsed with PBS and mounted with Prolong Gold Antifade reagent with DAPI (Invitrogen). Cells were observed under the Zeiss Fluorescence Microscope (Carl Zeiss AG) with the FITC and DAPI channels and imaged with the attached Canon

Powershot camera (Canon). Fluorescent cells on the images captured were counted with the ImagePro Software (Media Cybernetics, Bethesda, MD).

## 2.9 Flow Cytometry

Cell cycle analysis of Hep 3B transiently co-transfected with plasmid DNA and siRNA was done with the fluorescence-activated cell sorter (FACS) to investigate the specificity of hnRNP K-knockdown induced apoptosis. Hep 3B cells seeded in 6-well plates at 60-70% confluency were co-transfected with 2 μg of pXJ40-HA or SDM-pXJ40-HA-hnRNP K-(a/b) together with 20 nM of si-ctrl or si-hnRNP K. At 72 h post transfection, cells were trypsinised with the use of 0.05% trypsin/ EDTA to obtain single-cell suspension and fixed with ice-cold 70% ethanol at least overnight. Subsequently, cells were washed with ice-cold PBS twice and treated with 100 μg/ml RNase A (Sigma-Aldrich) (Appendix I) for 5 min at room temperature. Treated cells were then stained with 50 μg/ml of propidium iodide (Sigma-Aldrich) (Appendix I) in dark for 1 h at room temperature. DNA content of stained cells was subsequently analysed with FACS Calibur and CellQuest software (BD Biosciences, Franklin Lakes, NJ).

## 2.10 Caspase Assays

### 2.10.1 Caspase Glo® 3/7 Assay

To examine the activity of caspase-3/7 in differentially transfected or treated Hep 3B and/ or HepG2 cells, Caspase Glo® 3/7 assay (Promega, Fitchburg, WI) was utilised and carried out according to manufacturer's instructions. Briefly, the total cell lysates of the differentially transfected or treated cells were first collected and quantitated as detailed in protein lysate extraction in Section 2.5.1. 10 μg of total cell

lysates in 50 µl of NP-40 cell lysis buffer from each reaction was loaded into each well of 96-well Flat Bottom Black Polystyrene Plates (Corning Inc. Lindfield, Australia) in triplicates on ice. 50 µl of Caspase Glo® 3/7 reagent was added into each well and incubated for 1 h at room temperature before caspase activity was measured with GloMax® 96 Microplate Luminometer (Promega) with manufacturer's pre-defined settings.

### 2.10.2 Caspase Glo® 8 Assay

To examine the activity of caspase-8 in differentially transfected and treated Hep 3B and HepG2 cells, Caspase Glo® 8 assay (Promega, Fitchburg, WI) was utilised and carried out according to manufacturer's instructions. Briefly, the total cell lysates of the differentially transfected or treated cells were first collected and quantitated as detailed in protein lysate extraction in Section 2.5.1. 10 µg of total cell lysates in 50 µl of NP-40 cell lysis buffer from each reaction was loaded into each well of 96-well Flat Bottom Black Polystyrene Plates (Corning Inc.) in triplicates on ice. Proteasome inhibitor MG-132 provided by the kit was first added to the Caspase-Glo® 8 reagent before 50 µl of the mixture was added into each well. Following incubation for 2 h at room temperature, caspase activity was measured with GloMax® 96 Microplate Luminometer (Promega) with manufacturer's pre-defined settings.

## 2.11 Chemical treatments

### 2.11.1 5-Fluorouracil (5-FU) treatment

Effects of 5-FU HCC cell viability

To investigate the effect of 5-FU on HCC cell viability, Hep 3B and HepG2 were seeded at concentration of 1.5-2 × $10^4$ cells/well in 100 µl DMEM/ 10% FBS in

triplicates in 96-well plates. Following overnight incubation, medium was removed and replaced with 20-100 μg/ml of 5-FU (Sigma-aldrich) dissolved in DMEM/ 10% FBS. Cells were treated for up two days and cell viability was measured at each time point with WST-1 assay as detailed in Section 2.7.

Effect of 5-FU on hnRNP K and p53 expression

To investigate the effect of 5-FU on the expression of hnRNP K and p53 in HCC cell lines, the various HCC cell lines were first seeded in 6-well plates. Following overnight incubation where the cells obtain 50-60% confluency (i.e. Hep 3B, $0.65 \times 10^6$ cells/well; HepG2, $1.2 \times 10^6$ cells/well), medium was removed and replaced with 20-50 μg/ml of 5-FU (Sigma-aldrich) dissolved in DMEM/ 10% FBS. At different time points up to 48 h, treated cells were harvested for total cell lysate or RNA collection separately. Thereafter, these total cell lysates and RNA were subjected to Western blot analyses or real-time quantitative PCR with specific primers respectively. Similar process was carried out with colorectal cancer cell lines HCT116 (p53+/+) and HCT116 (p53-/-) that were plated at a concentration of $1.2 \times 10^6$ cells/well.

Examination of 5-FU induced hnRNP K downregulation

To determine whether the downregulation of hnRNP K following 5-FU treatment was a consequence of apoptosis induction, i.e. cleaved by caspases, Hep 3B, HepG2 and the pair of colorectal cancer cell lines (HCT116) were first seeded in 6-well plates. Following overnight incubation where the cells obtain 50-60% confluency (i.e. Hep 3B, $0.65 \times 10^6$ cells/ well; HepG2 and HCT116s, $1.2 \times 10^6$ cells/well), cells were first treated with 10 μM of pan-caspase inhibitor, Z-VAD-fmk (R&D Systems, Minneapolis, MN) or DMSO for 30 min prior the addition of 5-FU (Sigma-aldrich) in

DMEM/ 10% FBS to yield a working concentration of 20-50 μg/ml. At different time points up to 48 h, treated cells were harvested for total cell lysate. Thereafter, these total cell lysates were subjected to Western blot analyses or Caspase Glo® 3/7 Assay.

### 2.11.2 FasL treatment

To examine the functionality of the Fas signalling pathway in HCC cell lines, Hep 3B and HepG2 were first seeded in 6-well plates. Following overnight incubation where the cells obtain 50-60% confluency (i.e. Hep 3B, $0.65 \times 10^6$ cells/well; HepG2, $1.2 \times 10^6$ cells/well), medium was removed and replaced with 2-100 ng/ml of FasL (Enzo Life sciences, Inc., Farmingdale, NY, cat. no.ALX-522-020-C005) in DMEM/ 10% FBS. At different time points up to 8 h post-treatment, cells were harvested for total cell lysate and subsequently subjected to Caspase Glo® 3/7 Assay.

### 2.11.3 TRAIL treatment

Examination of TRAIL signalling in HCC cells

To examine the functionality of the TRAIL signalling pathway in HCC cell lines, Hep 3B and HepG2 were first seeded in 6-well plates. Following overnight incubation where the cells obtain 50-60% confluency (i.e. Hep 3B, $0.65 \times 10^6$ cells/well; HepG2, $1.2 \times 10^6$ cells/well), medium was removed and replaced with 2-50 ng/ml of TRAIL (Enzo Life sciences, Inc., cat. no.ALX-201-115-C010) in DMEM/ 10% FBS. At different time points up to 8 h post-treatment, cells were harvested for total cell lysate and subsequently subjected to Caspase Glo® 3/7 Assay.

Effect of hnRNP K downregulation or overexpression on TRAIL-induced caspase activity

To examine the effect of hnRNP K reduction or overexpression on TRAIL-induced caspase activity in HCC cell lines, Hep 3B and HepG2 were first seeded (i.e. Hep 3B, $0.65 \times 10^6$ cells/well; HepG2, $1.2 \times 10^6$ cells/well) and transfected with either siRNA or hnRNP K expression plasmids in 6-well plates. Following 24 h of transfection, medium was removed and replaced with 2 ng/ml of TRAIL in DMEM/ 10% FBS. At 2 h and 8 h post-treatment, cells were harvested for total cell lysate and subsequently subjected to Caspase Glo® 3/7 Assay and Caspase Glo® 8 assay (for hnRNP K reduction experiment).

To examine the profiles of TRAIL-induced caspase-3/7 activity in HepG2 cells treated with si-ctrl and si-hnRNP K, differentially transfected HepG2 cells were treated with 2-50 ng/ml of TRAIL for 8 h before caspase-3/7 activity was measured.

Determination of BID levels in hnRNP K downregulation enhanced TRAIL-induced caspase activity

To examine whether BID cleavage contributed to the enhanced TRAIL-induced caspase activity following hnRNP K reduction, HepG2 was seeded at a concentration of $1.2 \times 10^6$ cells/well in 6-well plates overnight. Medium was removed and replaced with 2-50 ng/ml of TRAIL in DMEM/ 10% FBS. At 8 h post-treatment, cells were harvested for total cell lysate and levels of full length BID were determined with Western blot analyses. Levels of full length BID were also determined in total cell lysates from Hep 3B and HepG2 differentially transfected and treated with siRNA and TRAIL with Western blot.

### 2.11.4 Actinomycin D treatment

To determine whether hnRNP K affects the expression of XIAP at the transcriptional or post-transcriptional level, Hep 3B cells were seeded and transfected with either 40 nM of si-ctrl or si-hnRNP K. Following 48 h of transfection, 5 μg/ml Actinomycin D (Sigma-Aldrich, Cat. No. A9415) was added to inhibit the *de novo* mRNA synthesis and incubated for the indicated periods of time. Total RNA was extracted and the levels of XIAP expression were determined with real-time quantitative PCR with specific primers.

## 2.12 Whole- genome Exon Array Analysis

40 nM of si-ctrl or si-hnRNP K were separately transfected into Hep 3B cells and the total RNA was extracted 24 h and 48 h post-transfection. 1 μg of total RNA was used as initial material for GeneChip Human Exon 1.0 ST Arrays (Affymetrix) according to manufacturer's instructions. After washing and staining of hybridised arrays with an Affymetrix Fluidics Station, they were scanned on Gene Array Scanner 2500 (Affymetrix) to capture the raw probe signal intensities in CEL files.

Preliminary data analysis was done by Dr. Stanley Ng Kwong Loong (Singapore Immunology Network). Briefly, background subtraction, quantile normalisation, and summarizing probe sets from Affymetrix expression microarrays raw CEL files were done with Affymetrix Power Tools (APT) software at the gene level and the resulting probesets were annotated with Affymetrix annotation library file (HuEx-1_0-stv2.na30.hg19.probeset.csv). With resultant probeset signals in log2 scale, log2 fold-change was calculated by subtracting the log2 gene expression of the appropriate controls (si-ctrl at each time point) from that of the treated sample (si-hnRNP K at each time point).

## 2.13 Statistical Analyses

All functional tests were done in triplicates and repeated at least twice with similar results obtained. Data shown as mean ± SD was from triplicates of a representative experiment. Differences of averages and percentages between the different plasmid/ siRNA transfectants or treatments were statistically analysed with Student's *t*-test. *p*-value lesser than 0.05 was considered to be statistically significant (*: $p<0.05$; **: $p<0.001$). For data from flow cytometry, 10,000 events were counted per sample.

# CHAPTER 3

## hnRNP K regulates survival in HCC cell lines

## 3.1 Introduction

As a member of the hnRNP family, hnRNP K is a multifunctional protein involved in many important biological processes that include transcription, translation and mRNA splicing (Bomsztyk et al., 2004). It has also been shown to play a role in cell proliferation (Ostrowski and Bomsztyk, 2003).

While hnRNP K has been shown to be involved in many fundamental biological processes, expression levels of hnRNP K have been reported to vary depending on cell type and developmental stage, e.g. hnRNP K is not ubiquitously expressed in all normal mouse tissues (Kamma et al., 1995) and in a rat development study, there was no detectable hnRNP K expression at certain stages of embryonic development in some tissues and organs (Blanchette et al., 2006). In addition, silencing of hnRNP K in *X. laevis* two-cell stage embryos inhibited axonal outgrowth but not cellular proliferation (Liu et al., 2008), raising the possibility that hnRNP K may possess additional roles other than its known function in cell proliferation.

In contrast to normal cells in development, hnRNP K has been reported to be upregulated in SV40-transformed cells (Dejgaard et al., 1994) as well as in various cancers studied (Table 3.1). In HCC, multiple studies have reported increased hnRNP K levels in tumour tissues compared to non-tumour tissues. Notably, an in-depth study reported elevated hnRNP K expression levels in early- and late-stage HCC as compared to cirrhotic liver samples (Guo et al., 2012). Thus, it could be hypothesised that hnRNP K has a role in cancer, given the significant difference in its expression levels observed between normal and cancer cells. In fact, hnRNP K has been reported to positively regulate several oncogenes as well as tumour suppressors, further suggesting its complex role in tumourigenesis (Michelotti et al., 1996; Moumen et al., 2005; Ritchie et al., 2003; Takimoto et al., 1993; Thakur et al., 2003).

**Table 3.1 List of human cancers reported with upregulated hnRNP K.**

| Type of human cancer | References |
|---|---|
| Breast Cancer | Mandal et al. (2001) |
| Colorectal Cancer | Klimek-Tomczak et al. (2006) |
| Esophageal Cancer | Hatakeyama et al. (2006) |
| Hepatocellular Carcinoma | Bai et al. (2009); Blanc et al. (2005); Guo et al. (2012); Kim et al. (2003); Li et al. (2004); Ostrowski and Bomsztyk, (2003) |
| Leukaemia (CML) | Perrotti and Neviani, (2007) |
| Lung Cancer | Pino et al. (2003) |
| Melanoma | Wen et al. (2010) |
| Nasopharyngeal Carcinoma | Chen et al. (2008a) |
| Pancreatic Cancer | Zhou et al. (2010) |
| Prostate Cancer | Nagano et al. (2004) |

Intriguingly, when chemotherapeutic agents were used to induce cancer cell death, e.g. mitomycin C, RITA, docetaxel and bicalutamide, it was observed that hnRNP K expression levels were reduced (Barboro et al., 2009; Benelli et al., 2009; Enge et al., 2009; Rahman-Roblick et al., 2007). Hence, there appears to be an association between hnRNP K levels and cancer cell survival. Indeed, a recent study conducted on colorectal cancer cell lines suggested the use of hnRNP K as a predictive marker to indicate response to 5-Fluorouracil (5-FU). Given that 5-FU is a first-line chemotherapeutic drug for HCC, the effect of 5-FU on hnRNP K levels was also examined in this study.

However, when the effect of hnRNP K downregulation in cancer cells was examined in various studies, the results were disparate. Reduction of hnRNP K via addition of siRNA has been reported to induce apoptosis in HeLa cells, HT29 colorectal adenocarcinoma cell line and Sy5y neuroblastoma cell line (Chen et al, 2010; White et al., 2010), but not in nasopharyngeal carcinoma cell lines and immortalised MRC5 lung fibroblast cell line (Moumen et al., 2005). However, it is to be noted that there were variations in experimental conditions across these studies, and that the extent of cell survival or death over the entire experimental period was neither tracked nor discussed. Thus, while hnRNP K has been reported to be associated with apoptosis by a few studies, the discordant findings highlight the need for further investigation into its role in apoptosis, i.e. if its downregulation induces apoptosis. Conversely, as hnRNP K is reported to be upregulated in HCC, it is of interest to investigate if this upregulation of hnRNP K plays a role in conferring increased resistance to apoptosis.

### 3.2 hnRNP K is found abundantly in HCC, predominantly in the nucleus

Given that hnRNP K was reported to be upregulated in HCC (Bai et al., 2009; Blanc et al., 2005; Kim et al., 2003; Li et al., 2004; Ostrowski and Bomsztyk, 2003), a panel of 17 HCC cell lines were examined for mRNA and protein levels of hnRNP K. In addition, as strong immunohistochemical hnRNP K staining has been demonstrated in HepG2 that alludes to its high expression levels (Uhlen et al., 2010) (Figure 1.6), it was of interest to investigate whether the other HCC cell lines in the panel also possess similarly high levels of hnRNP K.

mRNA levels of hnRNP K in HCC cell lines were first examined, where total RNA was extracted and reverse-transcribed to obtain cDNA, followed by PCR amplification using *HNRNPK* and *GAPDH* primers as detailed in Chapter 2. The primers were able to amplify both isoforms of hnRNP K, which could be distinguished by differences in their amplicon length. As shown in Figure 3.1, both isoforms of hnRNP K were detected in abundance in the panel of HCC cell lines tested, except for HepG2 that had low mRNA expression levels of both isoforms (Figure 3.1A) despite the strong hnRNP K staining reported by Uhlen et al. (2010). It could also be observed that the expression level of isoform b was generally lower as compared to isoform a. Possible mutation(s) in the coding region of hnRNP K in HepG2 was ruled out as the use of different primer sets amplifying different regions of hnRNP K gave similar results for HepG2 (Appendix II Figure 1).

On the contrary, when hnRNP K protein levels were examined in the total cell lysates of the HCC cell lines (Figure 3.1B), the protein expression levels of hnRNP K were comparable and present in abundance across all HCC cell lines. No particular HCC cell line showed a lack of hnRNP K protein expression. Hence, this indicated that the low hnRNP K mRNA expression level in HepG2 did not correlate with its

hnRNP K protein expression level. This could be due to the increased stability of hnRNP K protein or rapid turnover of hnRNP K mRNA in HepG2 cells, which could be focused on in future studies. Nonetheless, the abundance of hnRNP K protein found in all HCC cell lines is in agreement with previous studies that reported hnRNP K upregulation in HCC cells.

In addition to the mRNA and protein expression levels of hnRNP K, the subcellular localisation of hnRNP K in HCC cells was examined in Hep 3B cells with the use of immunofluorescence microscopy. hnRNP K was observed to locate predominantly in the nuclei (Figure 3.2), which is in agreement with a study by Guo et al. (2012) that reported elevated hnRNP K nuclear expression levels in early- and late-stage HCC, as well as that described in HepG2 by Uhlen et al. (2010) (Figure 1.6).

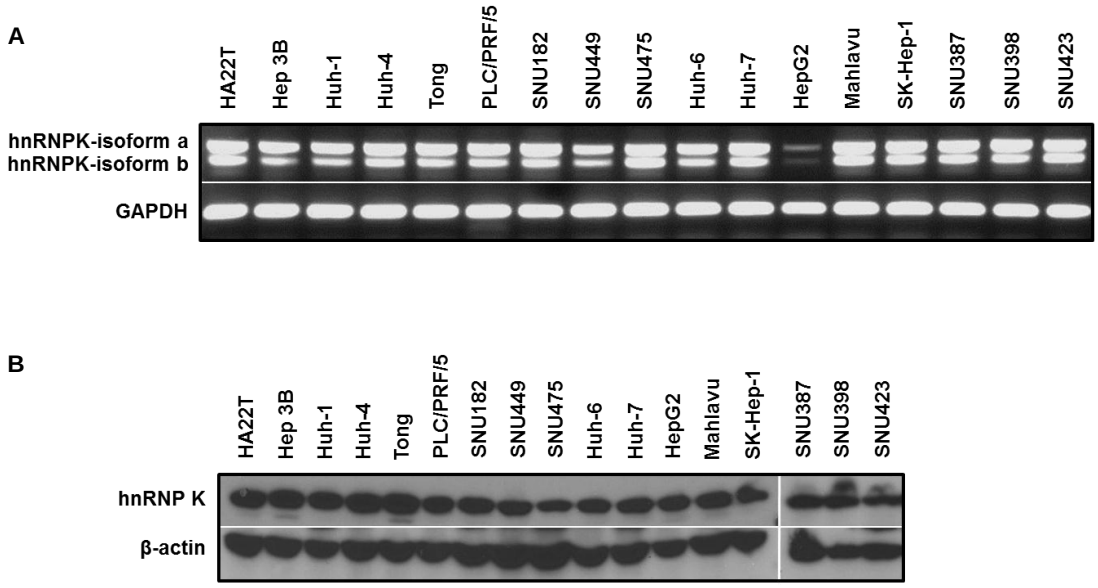


**Figure 3.1 Expression levels of hnRNP K in a panel of 17 HCC cell lines**
**(A)** mRNA expression levels of hnRNP K isoform–a and –b in 17 HCC cell lines. Total RNA was extracted from the 17 HCC cell lines, reverse-transcribed to form cDNA and subjected to subsequent PCR. Primers specific for hnRNP K were utilised for PCR to amplify the two hnRNP K isoforms and primers specific for GAPDH were also used for PCR to serve as loading control. **(B)** Western blot analyses revealed that hnRNP K protein was found in abundance in all HCC cell lines. 20 μg of total cell lysate was loaded into each lane and blotted with polyclonal antibody against hnRNP K. The antibody used was against common amino acids present in both isoforms and not isoform-specific. The blot was stripped and re-blotted with monoclonal antibody against β-actin that served as a loading control.

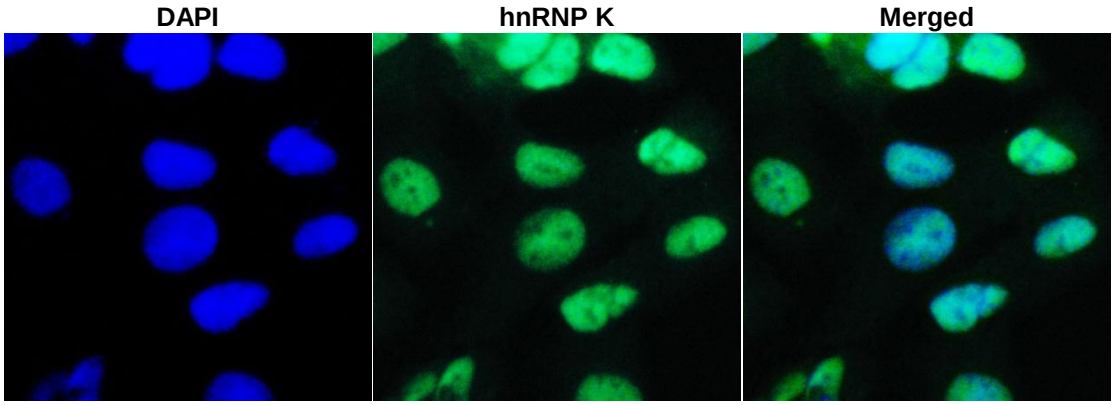


**Figure 3.2 hnRNP K localises predominantly in the nucleus of HCC cells**
Cultured Hep 3B cells were fixed, permeabilised and subjected to immunofluorescence staining. The localisation of hnRNP K (green) strongly correlated with DAPI nucleic acid stain (blue), revealing that hnRNP K is located predominantly in the nucleus of Hep 3B cells.

## 3.3 p53-independent regulation of HCC cell survival

### 3.3.1 5-Fluorouracil affects HCC cell viability and downregulates hnRNP K independently of p53

Upon addition of chemotherapeutic drugs that decreased cancer cell viability, hnRNP K expression level was observed to be reduced (Barboro et al., 2009; Benelli et al., 2009; Enge et al., 2009; Rahman-Roblick et al., 2007). Coupled with the suggestion that hnRNP K could serve as a predictive marker indicating response to 5-FU (Zhang et al., 2010), it was therefore of interest to examine the effect of 5-FU on hnRNP K expression. 5-FU is a commonly used anti-metabolite chemotherapeutic drug to treat various cancers but its usage has been limited due to the development of multi-drug resistance; nonetheless it is accepted as a first-line anti-cancer drug for HCC chemotherapy and has been used in combination with other agents such as leucovorin and interferon-α in the chemotherapeutic treatment of advanced unresectable HCC (Abdel-Hamid and Morsy, 2010; Patt et al., 2003; Porta et al., 1995; Stuart, 2012; Tetef et al., 1995; Tong et al., 2012; Uchibori et al., 2012). As 5-FU induces the upregulation of p53 at the protein level (Ju et al., 2007) and is associated with p53-dependent apoptosis (Enge et al., 2009), hence its effect on hnRNP K was examined in light of prior suggestions that hnRNP K is regulated by p53 (Mirza et al., 2003; Rahman-Roblick et al., 2007).

Viability of HCC cells was first examined with the use of WST-1 assay following exposure to different doses of 5-FU. Commonly used HCC cell lines Hep 3B and HepG2 were chosen for preliminary analysis as Hep 3B is a p53-null HCC cell line while HepG2 possesses wild-type p53 (Petitjean et al., 2007).

From Figure 3.3, it could be observed that 5-FU treatment reduced the viability of HCC cells regardless of p53. In Hep 3B, the percentage absorbance of cells treated with 20 ng/μl of 5-FU for two days was 20.6% that of dimethyl sulfoxide (DMSO)-

treated cells. In HepG2, the percentage absorbance of cells treated with 20 ng/µl of 5-FU for two days was 17.3% that of DMSO-treated cells. This is in agreement with a previous report that 5-FU induced apoptosis via p53-independent pathways in laryngeal squamous cell carcinoma (Liu et al., 2006). In addition, the reduced viability in p53-null Hep 3B was in agreement with previous studies where 5-FU has been demonstrated to induce apoptosis in this cell line (Koike et al., 2006; Ma et al., 2011). The greater reduction in cell viability of HepG2 cells as compared to Hep 3B cells observed at higher 5-FU doses could have been due to activation of wild-type p53 in HepG2.

Following the observation that HCC cell viability decreased upon 5-FU treatment, the protein expression levels of hnRNP K and p53 were examined via western blot in 12 HCC cells lines that were treated with 5-FU or DMSO over different time points.

The results revealed that 5-FU treatment resulted in an accumulation of p53 in HCC cell lines with wild-type p53 and to a certain extent, in cell lines with p53 mutants (Figure 3.4), which was consistent with a previous report by Ju et al. (2007). Interestingly, hnRNP K was observed to be downregulated in all 12 HCC cell lines upon 5-FU treatment regardless of their p53 status, indicating that the hnRNP K reduction was independent of p53. In addition, as 5-FU has been demonstrated to induce apoptosis in both p53–dependent and –independent manners (Enge et al., 2009; Liu et al., 2006), the experimental findings suggest that the reduction of hnRNP K following 5-FU treatment could be associated with apoptosis.

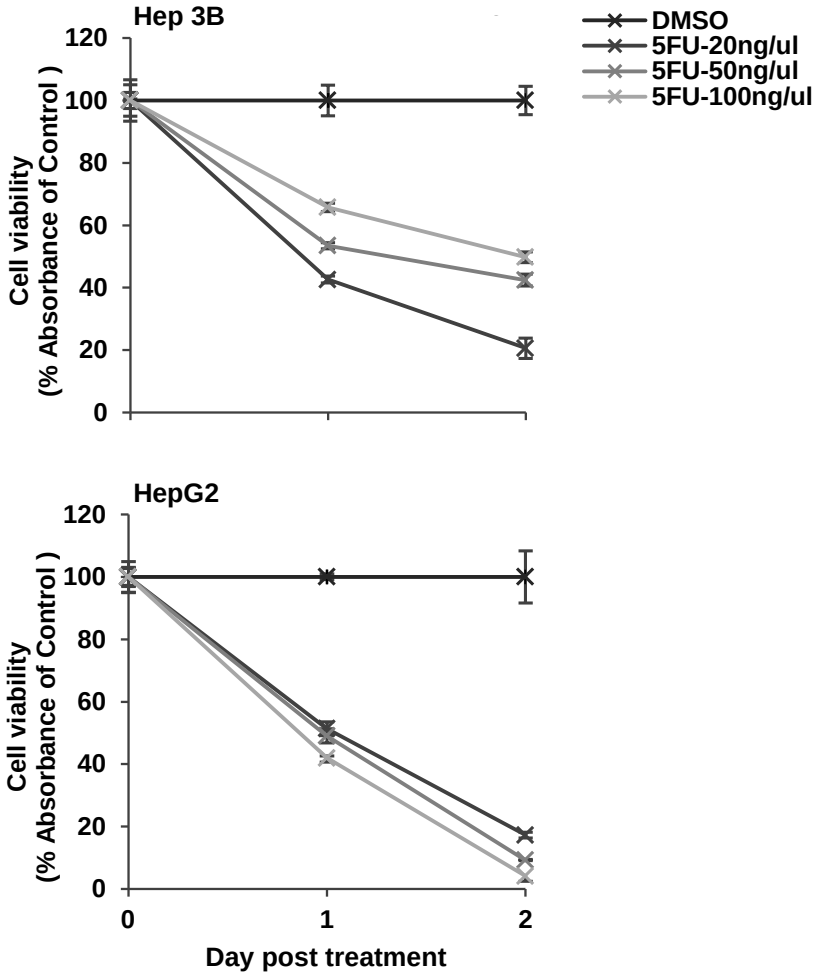


**Figure 3.3 5-Fluorouracil treatment reduces viability of HCC cells**

WST-1 cell viability results of Hep 3B and HepG2 cells (top and bottom panels respectively) treated with the indicated concentrations of 5-FU. Cells were treated with the indicated 5-FU concentration one day after they were plated into 96-well plates. Data presented as mean ± SD% absorbance of triplicates and expressed as the percentage absorbance relative to the absorbance of DMSO control at each time point. Results shown are from one representative experiment of at least two with similar trend.

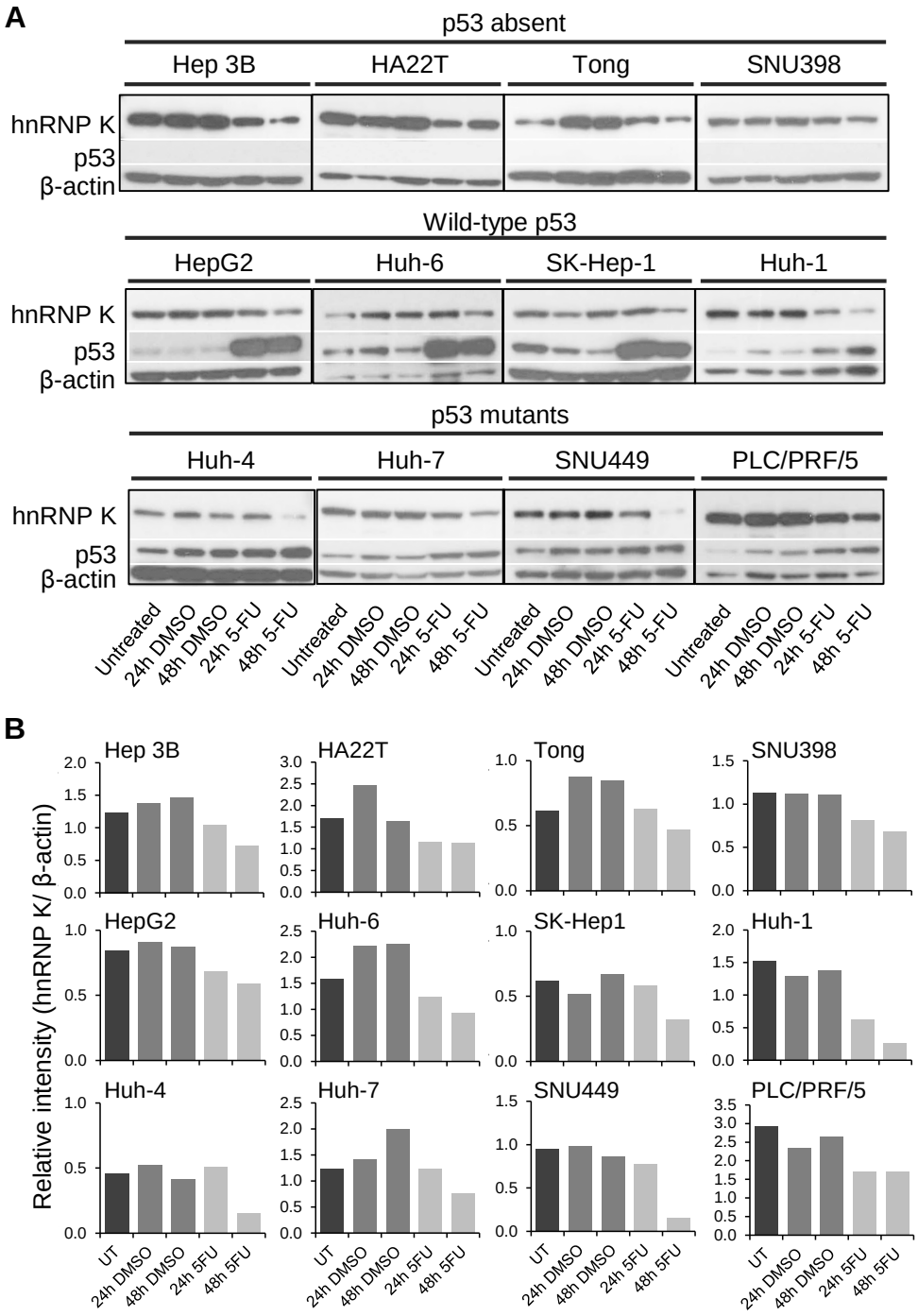


**Figure 3.4 5-Fluorouracil reduces hnRNP K in HCC cells regardless of p53 status**

12 HCC cell lines were treated with 20 ng/µl 5-Fluorouracil (5FU) or DMSO as indicated for 24 h or 48 h and subjected to western blot analyses for the examination of hnRNP K and p53 protein levels with the use of specific antibodies. β-actin was re-blotted and served as a loading control. 5-FU treatment stabilised and increased p53 protein level only in cells with p53 but decreased hnRNP K level across all HCC cell lines regardless of p53 status. **(A)** Western blot analyses for the HCC cell lines were displayed based on their p53 status. **(B)** Corresponding densitometry measurements for the expression levels of hnRNP K in the HCC cell lines.

### 3.3.2 Downregulation of hnRNP K by 5-Fluorouracil occurs at the mRNA level

In an attempt to demonstrate that 5-FU-induced downregulation of hnRNP K is not only restricted to HCC cells, colorectal cancer cells sensitive to both p53–dependent and –independent 5-FU induced apoptosis, HCT116 (p53+/+) and HCT116 (p53-/-) (Chan et al., 2008; Zhao et al., 2008) were examined in addition to Hep 3B and HepG2 cells. As revealed by the western blot analyses, 5-FU was able to downregulate hnRNP K in Hep 3B, HepG2 and both HCT116 cell lines in a time and dose-dependent manner (Figure 3.5). In addition, it could be further concluded that reduction of hnRNP K by 5-FU was p53-independent, given the reduction observed in both HCC and colorectal cancer cells.

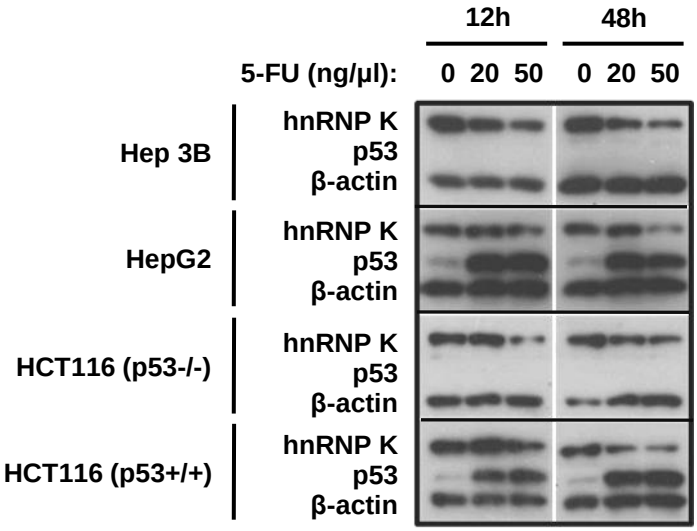


**Figure 3.5 5-Fluorouracil treatment downregulates hnRNP K in a dose- and time-dependent manner**

HCC cell lines Hep 3B and HepG2, as well as colorectal cancer cell lines HCT116 (p53+/+) and HCT116 (p53-/-) were treated with DMSO, 20 ng/µl or 50 ng/µl of 5-FU over time points of 12 h and 48 h. Total cell lysates were analysed with western blots for the levels of hnRNP K, p53 and β-actin that served as a loading control.

In previous studies, hnRNP K has been suggested to be a caspase substrate (Dix et al., 2008; Mahrus et al., 2008). Therefore, to investigate if the observed 5-FU-induced downregulation of hnRNP K was a consequence of apoptosis, i.e. due to cleavage by caspases, both pairs of HCC and HCT116 cell lines were pre-treated with either 10 μM of pan-caspase inhibitor or its control (DMSO) prior to 5-FU treatment. Following this, the protein levels of the hnRNP K and p53 from the differentially treated cells were examined with the use of western blot. Caspase-3/7 activity in these cells was also subsequently measured with Caspase-Glo® 3/7 Assay, in order to demonstrate the successful inhibition of caspases by the pan-caspase inhibitor.

Despite the addition of pan-caspase inhibitor, the results from Figure 3.6A revealed that hnRNP K downregulation induced by 5-FU persisted in a dose-dependent manner. In addition, as the results from the caspase-3/7 assay revealed the inhibition of caspase-3/7 activity by the pan-caspase inhibitor, this indicated that the observed hnRNP K downregulation was not a consequence of apoptosis, i.e. not cleaved by caspases (Figure 3.6B).

Without the addition of the pan-caspase inhibitor, increased caspase-3/7 activity was observed following 5-FU treatment, which concurred with previous reports that 5-FU treatment can result in p53–dependent and –independent apoptosis (Chan et al., 2008; Enge et al., 2009; Koike et al., 2006; Liu et al., 2006; Ma et al., 2011; Zhao et al., 2008).

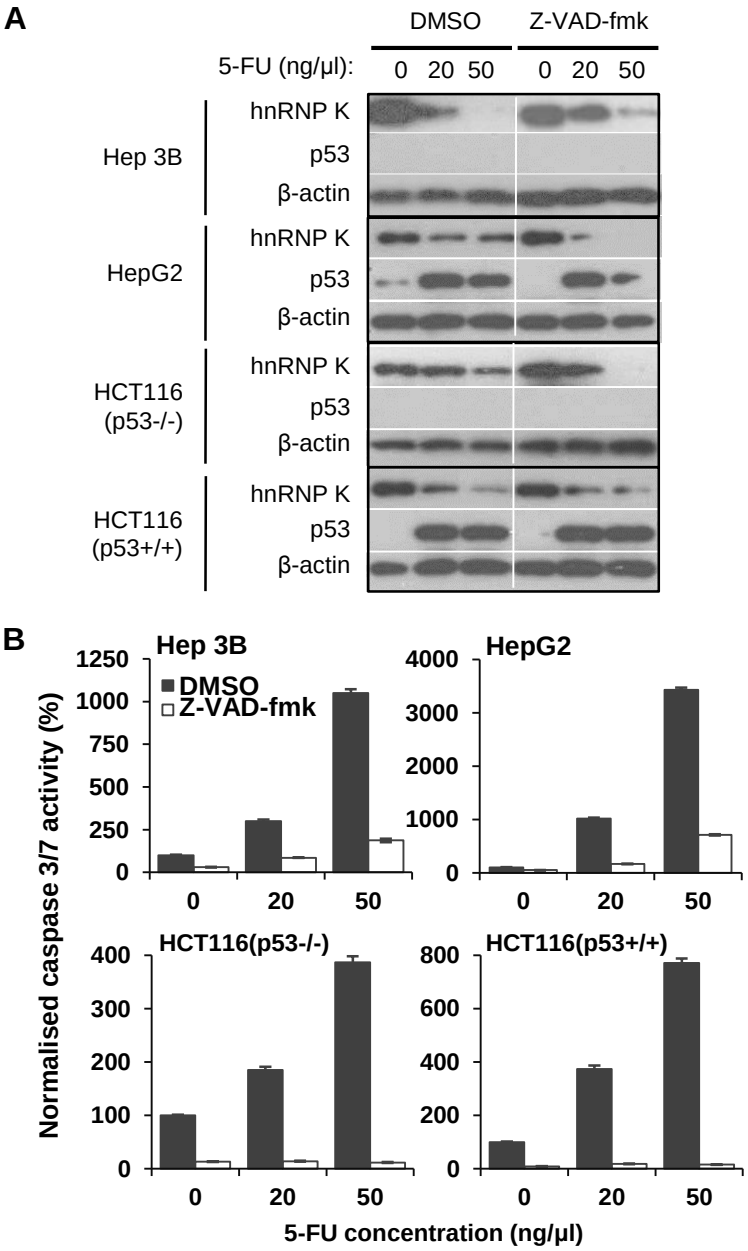


**Figure 3.6 5-Fluorouracil mediated downregulation of hnRNP K is independent of cleavage by caspase**

HCC cell lines (Hep 3B and HepG2) as well as colorectal cancer HCT116 cell lines (both p53+/+ and p53-/-) were pre-treated with 10 µM of pan-caspase inhibitor (Z-VAD-fmk) or DMSO solvent as control for half an hour before the addition of DMSO or 20 ng/µl or 50 ng/µl of 5-FU. Total DMSO concentration used was 0.15%. Total cell lysates were collected after 48 h of treatment and were subjected to **(A)** Western blot analyses of hnRNP K, p53 and β-actin that served as a loading control and **(B)** Caspase-Glo® 3/7 Assay as described in Chapter 2. Data presented as caspase-3/7 activity of cells relative to DMSO-treated cells as mean ± SD % of triplicates. Results shown are from a representative experiment of at least two with similar trend.

Having demonstrated that the 5-FU-induced hnRNP K downregulation observed is not a consequence of apoptosis, real-time quantitative PCR was subsequently utilised to examine whether the downregulation of hnRNP K by 5-FU occurs at the mRNA level. As shown in Figure 3.7, there was a decrease in hnRNP K mRNA level in both Hep 3B and HepG2 following 12 h and 24 h of 5-FU treatment, indicating that the hnRNP K downregulation after 5-FU treatment was likely due to a reduction in mRNA.

Taken together, the experimental findings from this current study show that 5-FU is a chemotherapeutic agent capable of downregulating hnRNP K at the mRNA level independently of p53, thus making hnRNP K likely one of the proteins that mediate a reduction in cell viability during 5-FU treatment.

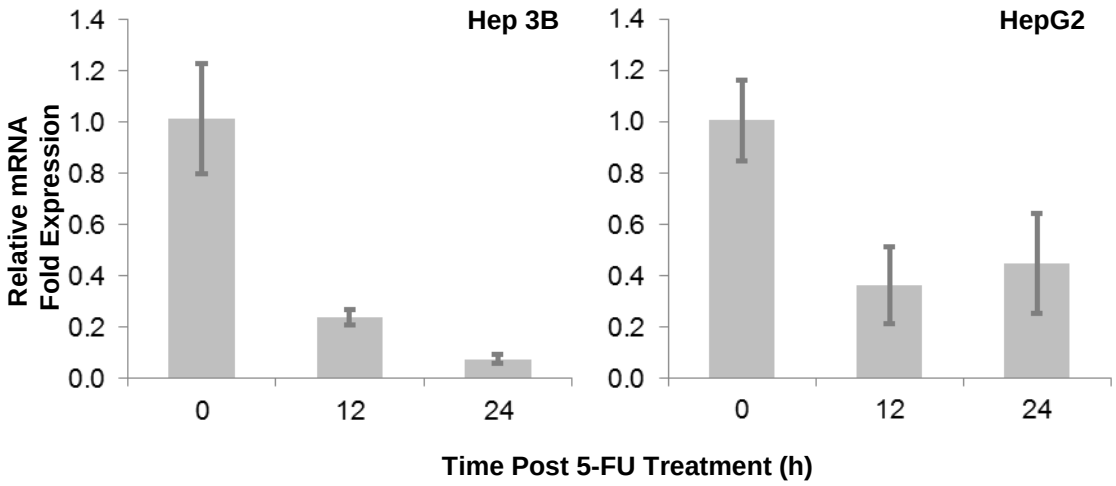


**Figure 3.7 5-Fluorouracil downregulates hnRNP K at the mRNA level**
Real-time quantitative PCR analyses of mRNA level of hnRNP K (both isoform–a and –b) in Hep 3B and HepG2 12 h and 24 h after treatment with 20 ng/µl of 5-FU. Data presented as mean ± SD of duplicates and expressed relative to untreated control after normalisation to *HPRT* expression level.

## 3.4 Effect of hnRNP K downregulation on cell morphology and viability

### 3.4.1 siRNA reduces hnRNP K expression levels effectively

In this study, addition of 5-FU has been demonstrated to downregulate hnRNP K levels, alongside a decrease in HCC cell viability. Thus, in order to determine whether a reduction in hnRNP K levels could be associated with decreased cell viability, as well as to investigate the role of hnRNP K in HCC, siRNAs were utilised to downregulate its expression in HCC cells.

To first determine the efficiency of hnRNP K downregulation by siRNAs, two siRNAs (hnRNP K siRNA 1 and 2) targeting different regions of the ORF that are common to both hnRNP K isoform–a and –b were transfected either individually or together into Hep 3B and HepG2 cells. Real-time quantitative PCR results revealed that the siRNAs could downregulate hnRNP K mRNA levels as early as 10h post-transfection, with an approximate reduction of 80% total hnRNP K mRNA as compared to the control (Figure 3.8A).

Western blot analyses of hnRNP K following siRNA transfection in the HCC cells revealed that it was significantly reduced at the protein level at 24 h post-transfection, which was more evident in Hep 3B than HepG2 (Figure 3.8B). hnRNP K level was observed to be at its lowest at 72 h post-transfection in both cell lines.

In addition, as hnRNP K localises predominantly in the nucleus of HCC cells, its subcellular localisation following siRNA treatment was examined in Hep 3B cells transfected with hnRNP K siRNA 1, which was more effective in downregulating hnRNP K at 48 h post-transfection (Figure 3.8B). siRNA treatment was observed to significantly decrease hnRNP K in the nucleus (Figure 3.9), suggesting that its nuclear functions, which include transcription and alternative splicing, are likely to be affected in HCC cells following its reduction with siRNA. Taken together, the results

reflected that the siRNAs specific against hnRNP K were effective in reducing hnRNP K mRNA and protein levels, with very significant protein level reduction in cells' nuclei.

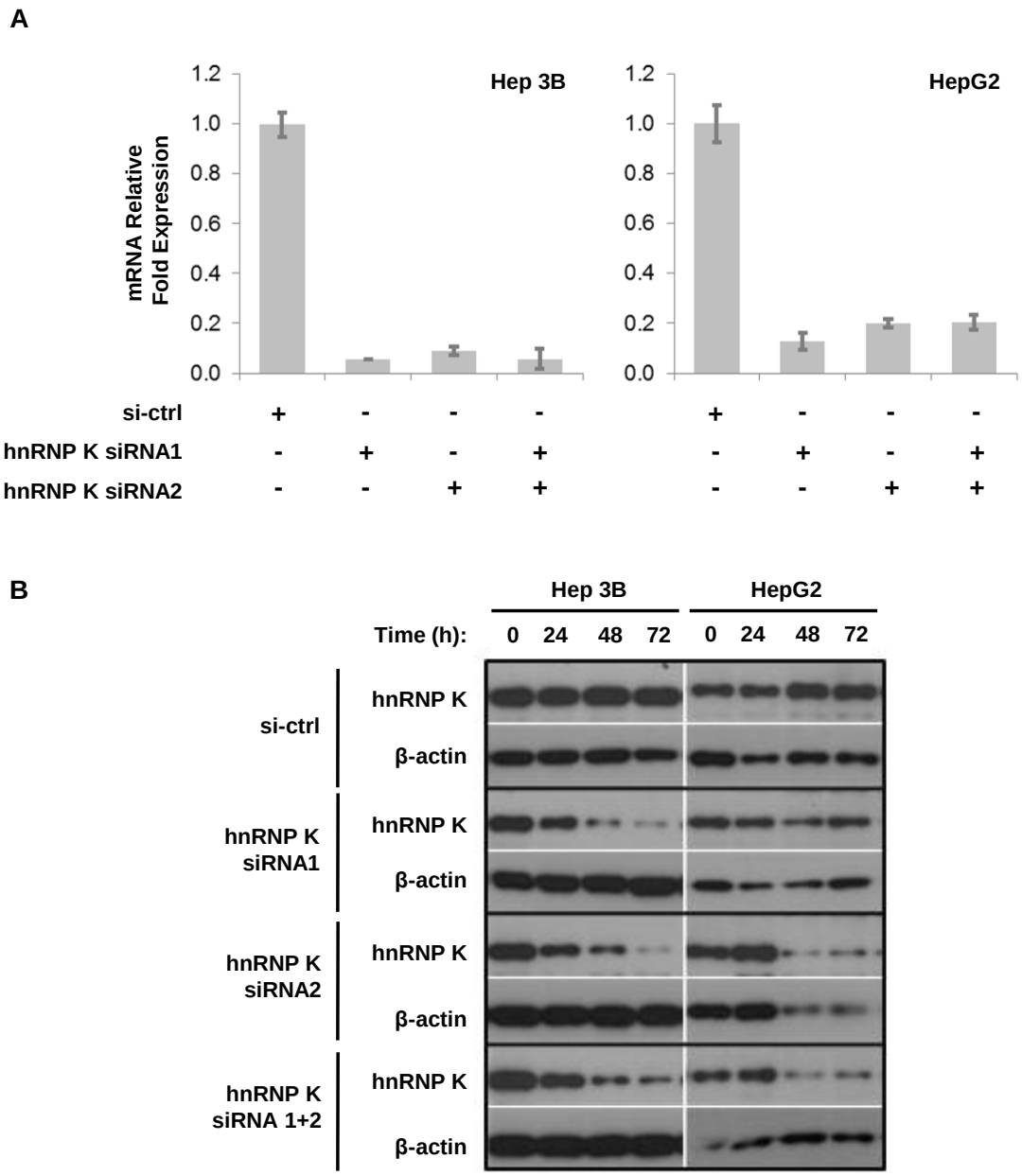


**Figure 3.8 siRNA treatment efficiently downregulates hnRNP K**

**(A**) Real-time quantitative PCR analyses of mRNA level of hnRNP K (all isoforms) in Hep 3B and HepG2 10h after transfection with siRNA specific against hnRNP K (40 nM). Data presented as mean ± SD of duplicates and expressed relative to cells transfected with non-specific control siRNA after normalisation to *HPRT* expression level. **(B)** Western blot analyses of hnRNP K protein level in total cell lysates of Hep 3B and HepG2 cells over different time point post- siRNA (40 nM) treatments with the use of polyclonal antibody against hnRNP K. Membranes were subsequently re-blotted with β-actin, which served as a loading control. **si-ctrl**, Non-specific control siRNA; **hnRNP K siRNA 1**, hnRNP K-specific siRNA Stealth Select RNAi$^{TM}$ siRNA (HSS179311); **hnRNP K siRNA 2**, hnRNP K-specific siRNA Stealth Select RNAi$^{TM}$ siRNA (HSS179312).

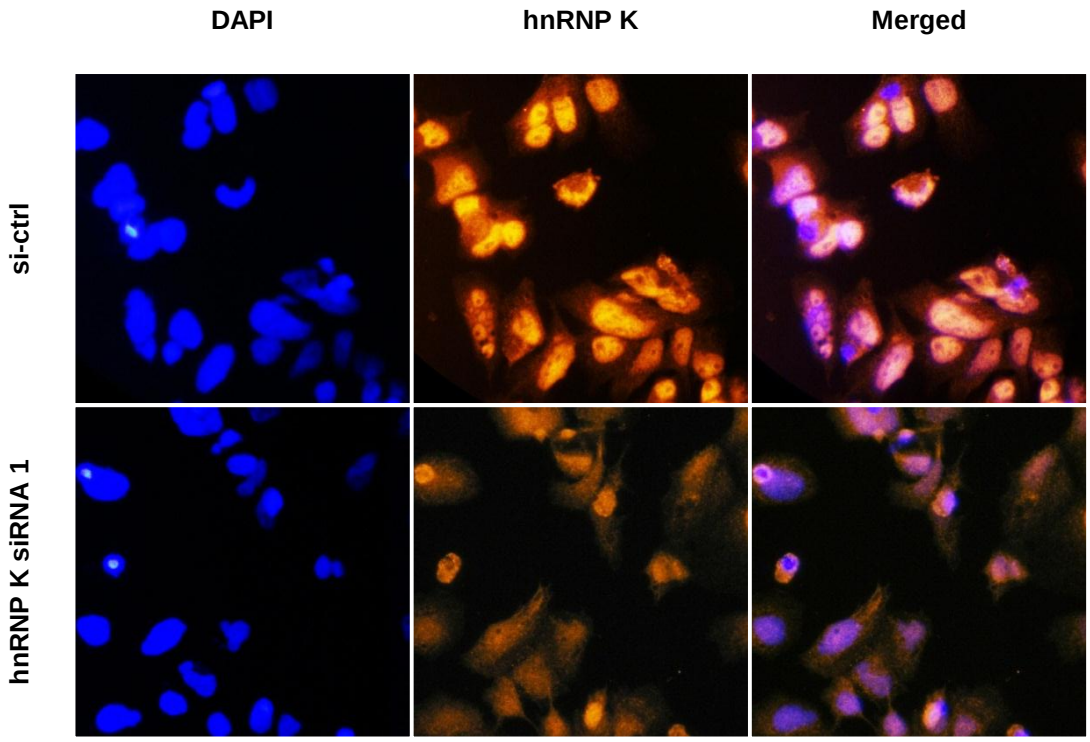


**Figure 3.9 Downregulation of hnRNP K with siRNA decreases its expression in the nucleus**

Hep 3B cells were transfected with 40 nM of either non-specific control siRNA or hnRNP K siRNA 1 for 48 h before immunofluorescence staining analysis of the subcellular localisation of hnRNP K. In Hep 3B cells transfected with si-ctrl, strong hnRNP K staining (Orange-red visualised with Rhodamine D channel) was observed to co-localise with DAPI staining (blue) in the nuclei while those transfected with hnRNP K siRNA 1 showed weaker hnRNP K staining and the residual hnRNP K staining could be observed in the cytoplasm. **si-ctrl**, Non-specific control siRNA; **hnRNP K siRNA1**, hnRNP K-specific siRNA Stealth Select RNAi$^{TM}$ siRNA (HSS179311).

### 3.4.2 hnRNP K reduction alters cell morphology and decreases cell viability

Following the demonstration that hnRNP K expression levels could be reduced effectively with siRNA treatment, the treated Hep 3B and HepG2 cells were also monitored for any phenotypic differences over time under phase-contrast microscope. The greatest visible cell number difference was observed at 96 h post-transfection, where control cells of Hep 3B attained approximately 90-100% confluency and HepG2 grew in clusters, while cells in both cell lines transfected with hnRNP K-specific siRNAs were scattered and lower in numbers (Figure 3.10A). A change in cell morphology was also observed at higher magnification; where both Hep 3B and HepG2 cells with hnRNP K-knocked down had 'bleb-like' structures and were rounder with clear cell boundaries as compared to control cells that possessed a 'polyhedral' shape and were close to neighbouring cells (Figure 3.10B). The findings indicate that the downregulation of hnRNP K expression levels in HCC cells caused cell morphology changes as well as reduced cell numbers.

In comparison to hnRNP K siRNA 2 as well as the combination of both siRNA 1 and 2, hnRNP K siRNA 1 resulted in more efficient reduction in both hnRNP K expression levels (Figure 3.8) and visible cell numbers (Figure 3.10). Hence, hnRNP K siRNA 1 (henceforth named as si-hnRNP K) was utilised for all subsequent functional analyses in this study.

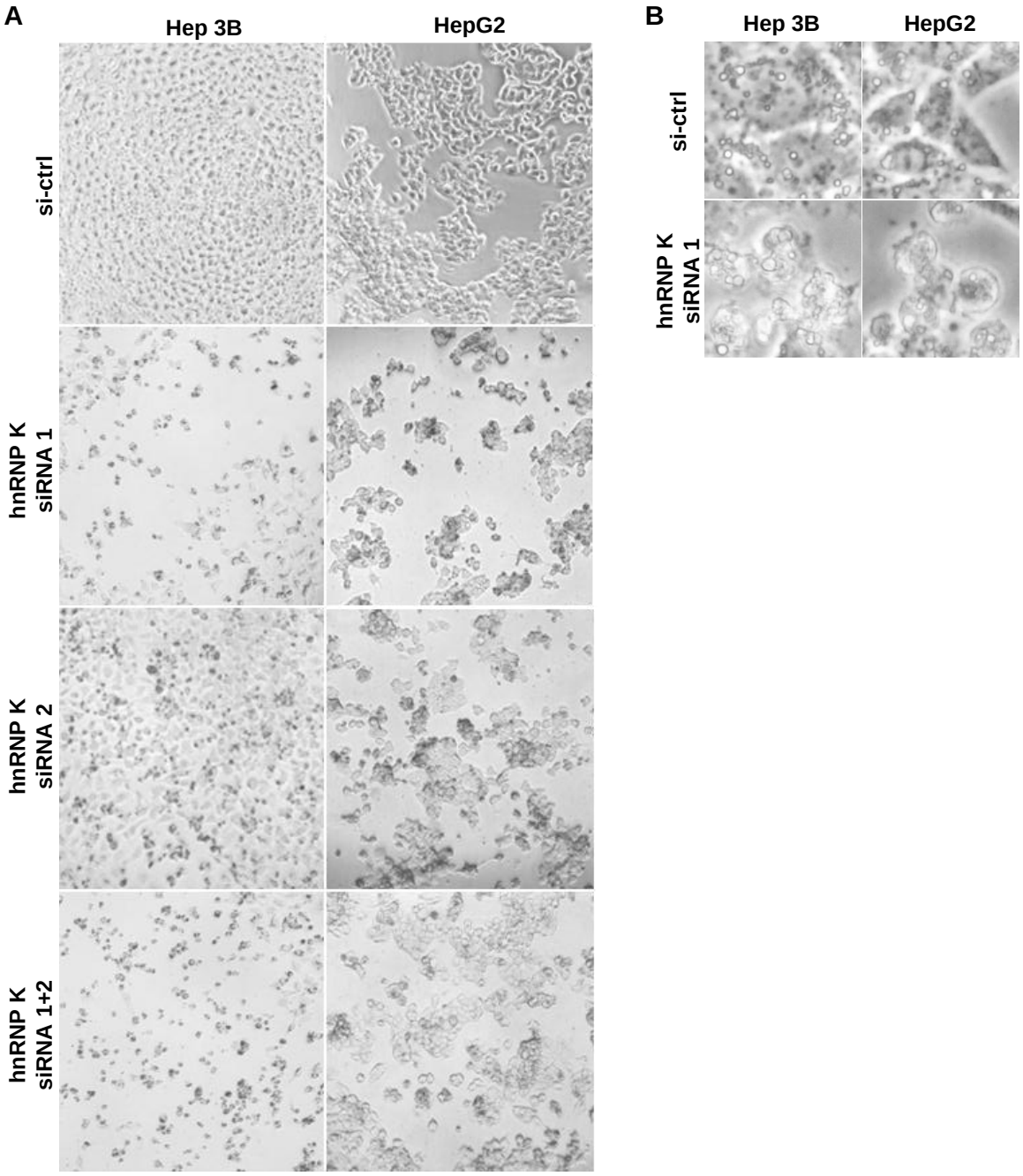


**Figure 3.10 Downregulation of hnRNP K results in a change of cell morphology**
Hep 3B and HepG2 cells were treated with control siRNA or hnRNP K specific siRNA(s) (40 nM) and observed under phase-contrast light microscope 96 h post transfection. **(A)** Knockdown of hnRNP K reduces visible HCC cell numbers. **(B)** Observation of HCC cells with hnRNP K downregulated at higher magnification revealed a change in cell morphology. **si-ctrl**, Non-specific control siRNA (si-control); **hnRNP K siRNA 1**, hnRNP K-specific siRNA Stealth Select RNAi$^{TM}$ siRNA (HSS179311); **hnRNP K siRNA 2**, hnRNP K-specific siRNA Stealth Select RNAi$^{TM}$ siRNA (HSS179312).

As a decrease in cell numbers was observed following hnRNP K reduction, the viability of HCC cells treated with si-hnRNP K was monitored closely over time with the use of WST-1 assay.

Consistent with prior observations made with the phase-contrast microscope (Figure 3.10), cell viability was significantly reduced in si-hnRNP K transfected cells as compared to control cells over time (Figure 3.11). At Day 4 post-transfection, the percentage absorbance of si-hnRNP K transfected Hep 3B and HepG2 was 17.7% ($p<0.05$) and 32.5% ($p<0.05$) of their control cells respectively (Figure 3.11B and C). A greater cell viability reduction was observed in Hep 3B as compared to HepG2 following siRNA treatment, which could be a result of the more significant reduction in hnRNP K expression in Hep 3B by si-hnRNP K as depicted in Figure 3.8B.

In summary, as presented with the use of Hep 3B and HepG2, the transient knockdown of hnRNP K in HCC cells resulted in a change in cell morphology and a pronounced reduction in cell viability, which could be due to an increase in cell death such as apoptosis.

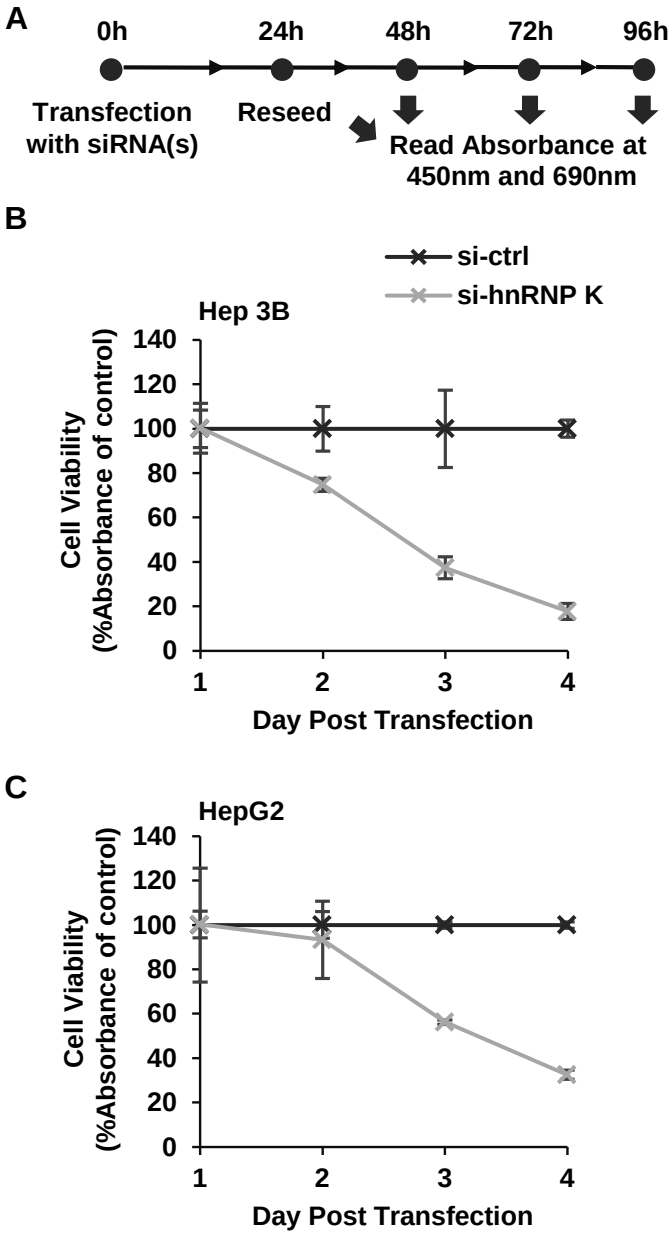


**Figure 3.11 Reduction of hnRNP K reduces cell viability**

**(A)** Experimental process for WST-1 viability and proliferation assay. Hep 3B and HepG2 cells were transfected with either si-control (si-ctrl) or si-hnRNP K in 6-well plates and reseeded into 96-well plates in triplicates 24 h later. Cell viability was monitored and measured daily. **(B)** and **(C)** WST-1 data of Hep 3B and HepG2 cells respectively transfected with si-control (dark grey) or si-hnRNP K (light grey). Cell viability data presented as mean ± SD% absorbance of triplicates and expressed as the percentage absorbance relative to control. Results are from a representative experiment of at least two with similar trend.

### 3.5 Downregulation of hnRNP K results in p53-independent apoptosis

Having demonstrated that the reduction of hnRNP K decreases cell viability, the role that hnRNP K could possibly play in apoptosis was investigated. There are various forms of programmed cell death, but the term 'apoptosis' has frequently been incorrectly used interchangeably with 'programmed cell death'. In fact, it has been recommended that various approaches should be employed to accurately classify a particular type of cell death as apoptosis instead of relying solely upon a single biochemical analysis method (Kroemer et al., 2009). Hence, various functional assays covering different biochemical features of apoptosis were used in this study to determine whether the decreased cell viability observed following hnRNP K downregulation was due to apoptosis. In addition, p53-null Hep 3B was chosen for experimental analyses of hnRNP K's role in apoptosis in order to exclude the contribution by p53, which is a well-known regulator of apoptosis (Fridman and Lowe, 2003).

To determine whether the reduced cell viability following hnRNP K downregulation was a result of apoptosis induction, the presence of caspase-cleaved PARP-1(poly ADP-ribose polymerase) was first determined in Hep 3B cells treated with si-hnRNP K using western blot. PARP-1, a 113kDa nuclear enzyme, is cleaved by caspase-3/7 during apoptosis to yield 24kDa and 89kDa fragments (Soldani and Scovassi, 2002), but is cleaved during necrosis to produce 55kDa and 62kDa fragments (Gobeil et al., 2001). Thus, to verify the induction of apoptosis, an antibody specific against the larger 89kDa caspase-cleaved PARP-1 fragment was utilised in western blot.

As depicted in Figure 3.12A, a decrease in the level of hnRNP K led to a corresponding increase in the level of caspase-cleaved PARP-1. The emergence of

PARP-1 cleavage in si-hnRNP K transfected Hep3B was specific, as cleaved PARP-1 was not detected in control cells. The results have indicated that apoptosis induction likely resulted in the reduced cell viability following hnRNP K reduction. In addition, the initial appearance of the 89kDa cleaved PARP-1 fragment was at 72 h post-transfection of Hep 3B cells with si-hnRNP K and featured most prominently at 96 h post-transfection. This suggested that the initiation of apoptosis was likely to be between 48 h and 72 h post-hnRNP K reduction as the cleavage of PARP-1 has been suggested to be an early critical biochemical event in apoptosis (Kaufmann et al., 1993).

Next, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was employed to further examine the induction of apoptosis by the knockdown of hnRNP K. Even though the labelling of cells that are necrotic or with extensive DNA damage have been reported (Grasl-Kraupp et al., 1995), TUNEL remains to be a commonly used method for labelling and identifying cells in the late phase of apoptosis by detecting oligonucleosomal DNA fragmentation (Kroemer et al., 2009; Negoescu et al., 1998; Negoescu et al., 1996).

Hep 3B cells with hnRNP K reduction for 96 h were subjected to TUNEL staining and the results in Figure 3.12B revealed a significant increase in TUNEL positive cells as compared to control. hnRNP K downregulated cells had 27.9 ± 4.5% TUNEL positive cells while control cells had only 3.8±1.2% TUNEL positive cells in the entire cell population ($p<0.05$). This observation also provide support that the downregulation of hnRNP K induces apoptosis.

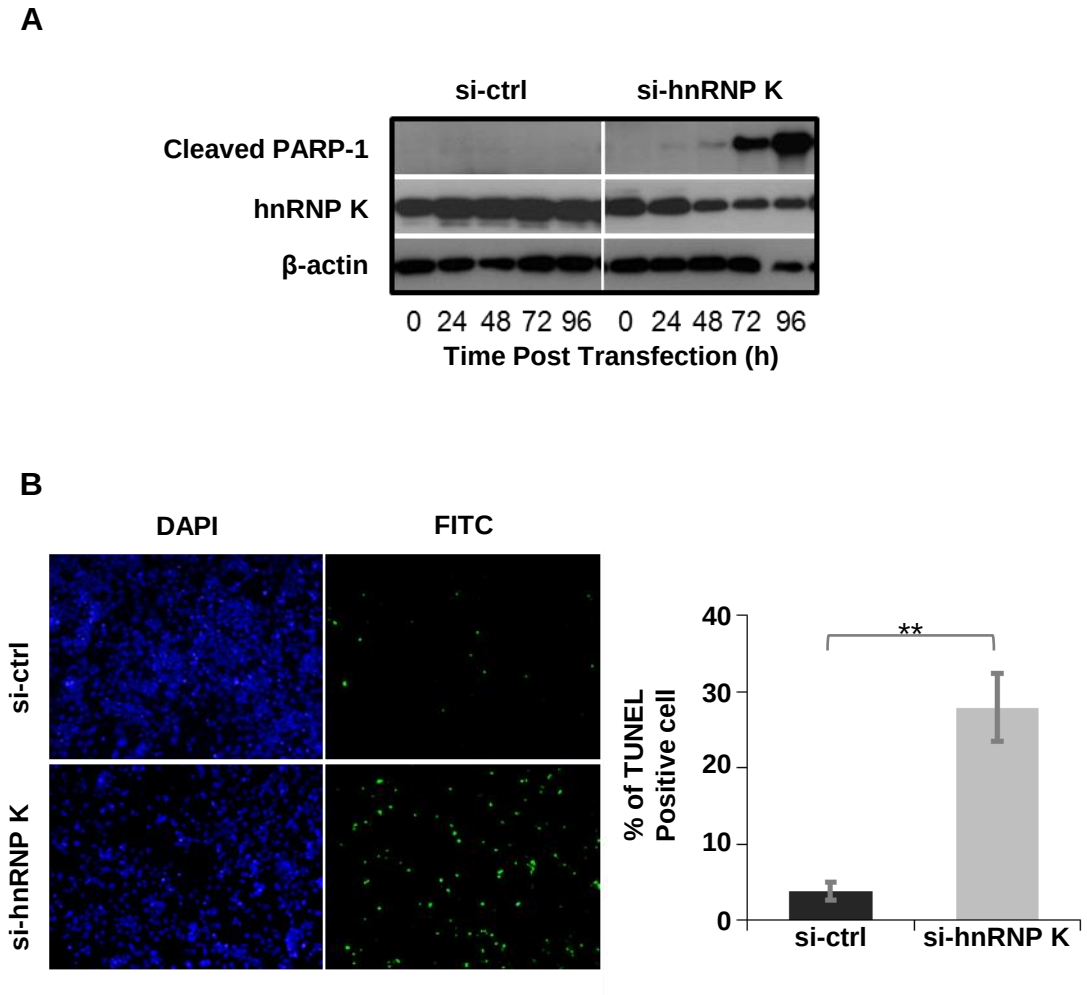


**Figure 3.12 hnRNP K downregulation induces apoptosis**

**(A)** Western blot analyses of hnRNP K protein level and cleaved PARP-1 in total cell lysates of Hep 3B over different time points post-siRNA (40 nM) treatments with the use of polyclonal antibody against hnRNP K and polyclonal antibody specifically against the 89kDa fragment of caspase-cleaved PARP-1. β-actin was re-blotted to serve as a loading control. **(B)** TUNEL assay revealed an increase in apoptotic cells following the downregulation of hnRNP K at 96 h post-transfection. The left panel shows the differentially transfected cells observed under the fluorescence microscope, where DAPI-stained cells represent the total cell population and FITC-stained cells represent TUNEL-positive cells that were undergoing apoptosis. DAPI-stained cells and TUNEL positive cells for each slide were counted with Image-Pro software. The right panel shows the percentage of TUNEL positive cells from Hep 3B transfected with si-control (dark grey) and si-hnRNP K (light grey) expressed as the mean ± SD percentages of FITC-stained cells of the total cell population (DAPI-stained) obtained from triplicates. (**$p<0.01$). Results are from a representative experiment of at least two with similar trend.

Apart from the detection of caspase-cleaved PARP-1 and TUNEL assay, activation of caspase is one of the most important determinants that identifies if cell-death was via apoptosis. Nevertheless, activation of caspases could also occur under non-lethal biological processes (Galluzzi et al., 2008). While caspases are activated in apoptosis, calpains and/or cathepsins are activated in necrosis (Kroemer et al., 2009). As such, having demonstrated that the knockdown of hnRNP K resulted in the production of cleaved PARP-1 and increased the number of TUNEL positive cells, it was of interest to examine the activation of the effector caspases.

To examine the activation of effector caspases over the course of hnRNP K reduction, Caspase-Glo® 3/7 assay was utilised to measure the activity of caspase-3/7. Total cell lysates of Hep 3B over the course of hnRNP K downregulation were collected and subjected to Caspase-Glo® 3/7 assay. As shown in Figure 3.13A, there was no significant difference in caspase-3/7 activity between hnRNP K downregulated and control cells at 24 h and 48 h post-transfection. However, a significant increase in caspase-3/7 activity was observed in cells with hnRNP K reduced as compared to control at 72 h post-transfection ($p<0.05$), and the observed increase in caspase-3/7 activity was the greatest at 96 h post-transfection ($p<0.05$) . Together with the TUNEL assay results as well as the detection of cleaved PARP-1, the above results have confirmed that the induction of apoptosis has contributed to the reduction in cell viability upon hnRNP K downregulation.

In addition, the significant increase in caspase-3/7 activity observed in Hep 3B with hnRNP K reduced as compared to control cells at 72 h and 96 h post-transfection was in concordance with the western blot analysis of cleaved PARP-1 protein level (Figure 3.12A), where induction of apoptosis was not detected in cells transfected with si-hnRNP K at 24 h and 48 h post-transfection, but only from 72 h post-

transfection onwards. These findings reflected that apoptosis is only induced following extended period of hnRNP K downregulation; hence suggesting that hnRNP K possibly functions as an anti-apoptotic protein in HCC, where its reduction increases the cells' susceptibility to apoptosis.

Having observed that apoptosis was induced following hnRNP K reduction in Hep 3B that is p53-null, indicating that the induction of apoptosis was p53-independent, the profile of caspase-3/7 activity in HepG2 cells with wild-type p53 following hnRNP K downregulation was similarly examined. Particularly, it was of interest to examine whether the presence of wild-type p53 in HepG2 could possibly contribute to the apoptosis induction following hnRNP K downregulation. As observed in Figure 3.13B, the profiles of caspase-3/7 activity in HepG2 was similar to that in Hep 3B over the course of hnRNP K downregulation (Figure 3.13A): there was no significant differences in caspase-3/7 activity between the differentially transfected HepG2 cells at 24 h and 48 h post-transfection, while caspase-3/7 activity was higher in cells with hnRNP K reduced at 72 h post-transfection ($p<0.01$) and this difference was the greatest at 96 h post-transfection ($p<0.01$). This thus suggested that the wild-type p53 that HepG2 possesses did not contribute to the apoptosis induced by the knockdown of hnRNP K.

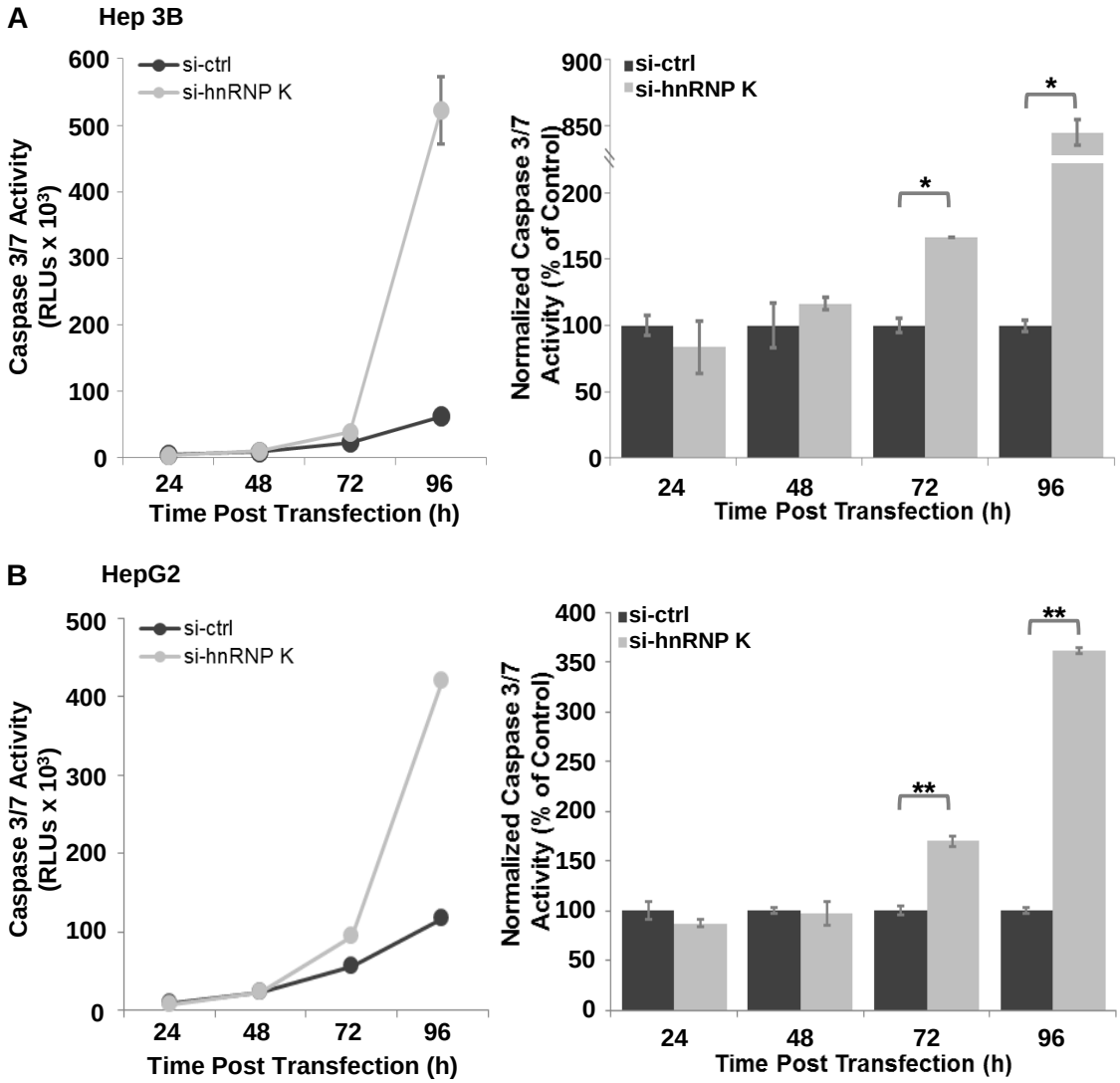


**Figure 3.13 Activation of caspase-3/7 following 72 h of hnRNP K reduction**

**(A)**Hep 3B and **(B)** HepG2 cells were transfected with either 40 nM of si-control (dark grey lines and bars) or si-hnRNP K (light grey lines and bars) over the different time points indicated; total cell lysates were collected and caspase-3/7 activities were measured with the use of Caspase-Glo® 3/7 assay. The left panels reflected the caspase-3/7 activities expressed as raw relative light units (RLUs) of triplicates while the right panels showed the relative caspase-3/7 activities of si-hnRNP K transfected cells after normalisation to si-control transfected cells at each time point stated and data were expressed as mean ± SD% of triplicates. (*$p<0.05$, **$p<0.01$). Results are from a representative experiment of at least two with similar trend.

To further establish that p53 was not involved in the apoptosis induction, it was knocked-down in HepG2 simultaneously with hnRNP K, and caspase-3/7 activity was determined over the course of downregulation. As shown in Figure 3.14A, it could be observed that the downregulation of p53 did not affect the activity of caspase-3/7 over the entire course of hnRNP K reduction. Real-time quantitative PCR results for 48 h and 72 h post-transfection had also demonstrated that the mRNA levels of p53 and hnRNP K were reduced by the siRNAs effectively (Figure 3.14B). The results thus verified the reduction of both proteins and indicated that the increased caspase-3/7 activity observed in cells with hnRNP K downregulation was contributed by the reduction of hnRNP K alone, without the involvement of p53.

Intriguingly, when HepG2 cells were transfected with a lower amount of si-hnRNP K (20 nM) as seen in Figure 3.14A, the profile and extent of caspase-3/7 activation over the course of its reduction was similar to those cells transfected with twice the amount of siRNA (40 nM) as seen in Figure 3.13B. Notably, apoptosis induction occurred at 72 h post-transfection, regardless of the amount of si-hnRNP K transfected. This hence indicated that the downregulation of hnRNP K most likely caused the cells to lose their ability to inhibit instead of actively inducing apoptosis.

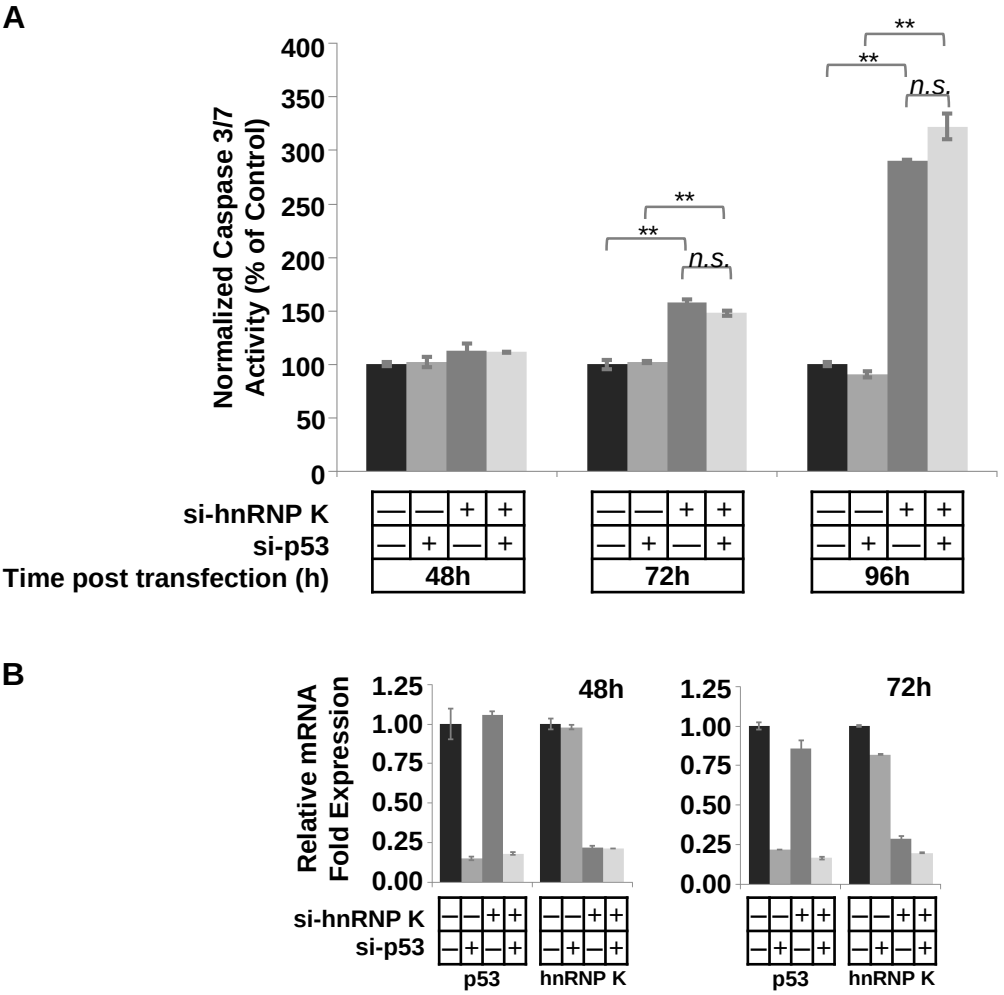


**Figure 3.14 Downregulation of hnRNP K induces p53-independent apoptosis**
HepG2 that possesses wild-type p53 was differentially transfected with the stated transfection combinations over different time points indicated. **(A)** Caspase-3/7 activities were measured and shown as normalised caspase-3/7 activity of each transfection combination relative to that transfected with si-control (both si-hnRNP K and si-p53 negative) at each time point. **(B)** mRNA levels of p53 and hnRNP K were examined in differentially transfected HepG2 with the with the use of real-time quantitative PCR with specific primers at 48 h and 72 h post-transfection and data were presented as mean ± SD of duplicates and expressed relative to cells transfected with si-control (both si-hnRNP K and si-p53 negative) after normalisation to *HPRT* expression level. (**$p<0.01$). si-p53, p53-specific siRNA. Transfection combinations comprise a total of 40 nM worth of siRNA where 20 nM of gene specific siRNA was used in each reaction and topped up with non-specific siRNA to 40 nM when required. Results are from a representative experiment of at least two with similar trend.

## 3.6 hnRNP K possess anti-apoptotic function

### 3.6.1 Testing for the specificity of hnRNP K in resisting apoptosis

Given that the downregulation of hnRNP K resulted in the onset of apoptosis in HCC cells, it was next investigated whether this apoptosis induced by its reduction was indeed hnRNP K-specific, i.e. whether restoring the levels of hnRNP K in these cells can reduce the extent of apoptosis induced.

To test the specificity of hnRNP K in resisting the onset of apoptosis, an experimental setup was designed as shown in Figure 3.15. Following the introduction of siRNA (si-hnRNP K), endogenous level of hnRNP K would presumably be downregulated. Concurrently, plasmids overexpressing hnRNP K would be introduced exogenously in an attempt to restore the reduced levels of the endogenous protein. Nonetheless, if these plasmids have DNA sequences identical to endogenous hnRNP K, they would likely be targeted and downregulated by the siRNA as well. Thus, to ensure that the levels of hnRNP K could be restored with the concurrent downregulation of the endogenous protein, silent mutations would have to be introduced into the sites that the siRNA targets on the plasmids. With this, functional assays for apoptosis can be subsequently carried out to compare cells with restored hnRNP K levels and those with its expression reduced.

To date, only two protein isoforms of hnRNP K have been described in detail, where isoform–a is slightly longer than isoform–b with key difference at the C-terminus. As the siRNA utilised (si-hnRNP K) targets a common region on the ORF of hnRNP K, both protein isoforms would presumably be downregulated. Hence, given the intention to restore hnRNP K levels with exogenously introduced plasmid expressing the protein, expression plasmids for both protein isoforms were constructed.

To create the constructs, the ORF of hnRNP K (isoform–a or –b) previously amplified from HepG2 and cloned into pcDNA3.1+ (Ng et al., 2005) were utilised as templates for PCR with designed primers. Purified PCR products were subsequently enzyme digested with *XhoI* and *SmaI* before being individually inserted into *XhoI*- and *SmaI*-digested pXJ40-HA vector downstream of both the CMV promoter ($P_{CMV}$) and HA (Hemagglutinin) epitope tag, as well as upstream of the SV40 polyadenylation site to produce the parental pXJ40-HA-hnRNP K (isoform–a or –b) constructs (Figure 3.16A).

After analysing the sequence of si-hnRNP K (Stealth Select RNAi$^{TM}$ siRNA HSS179311) as shown in Figure 3.16C, nucleotide positions 1182, 1188, 1197 and 1203 of hnRNP K ORF were chosen for site-directed mutagenesis to create silent mutations at amino acid positions 394, 396, 399 and 401 respectively. Being evenly spaced out on the target sequence of the ORF, these sites were chosen such that their mutation would disrupt the complementary base pairing with si-hnRNP K to prevent plasmid degradation. The DNA chromatographs of the pXJ40-HA-hnRNP K that underwent four rounds of step-wise sequential site-directed mutagenesis are shown in Figure 3.16D, which yielded the resultant SDM-pXJ40-HA-hnRNP K (isoform–a or –b) expression plasmids (Figure 3.16B). The expression plasmids harbouring the four silent mutations were subsequently examined for their ability to express hnRNP K when co-transfected with si-hnRNP K.

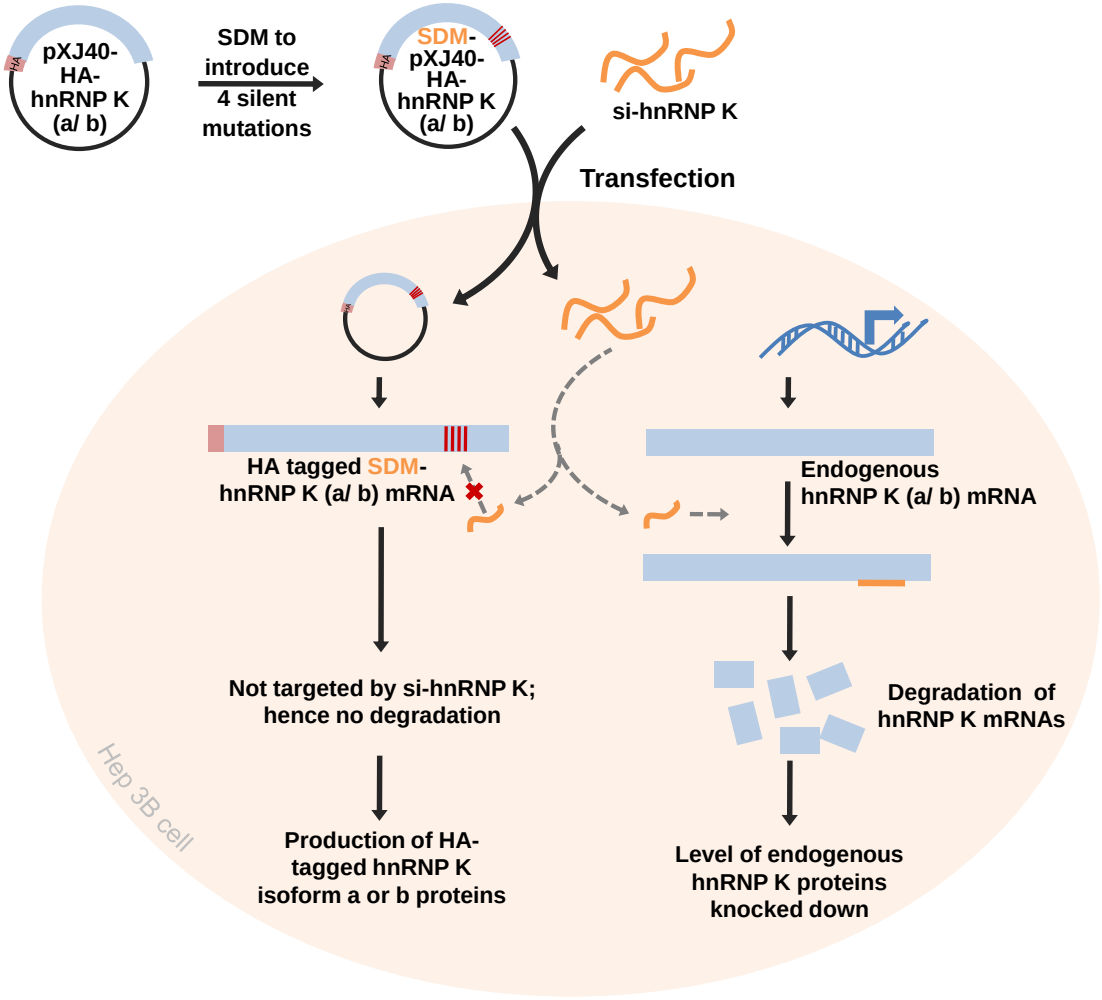


**Figure 3.15 Schematic workflow to test the specificity of hnRNP K in the apoptosis induced by its reduction**

In order to prevent hnRNP K expression plasmid from being targeted by si-hnRNP K for degradation so as to restore its protein levels, pXJ40-HA-hnRNP K–a or –b were subjected to site-directed mutagenesis to introduce four silent mutations to generate SDM-pXJ40-HA-hnRNP K–a or –b. As such, si-hnRNP K introduced into the cells will target endogenous hnRNP K for degradation while the exogenously introduced plasmids would be spared, thereby restoring the level of hnRNP K in cells for subsequent downstream functional studies.

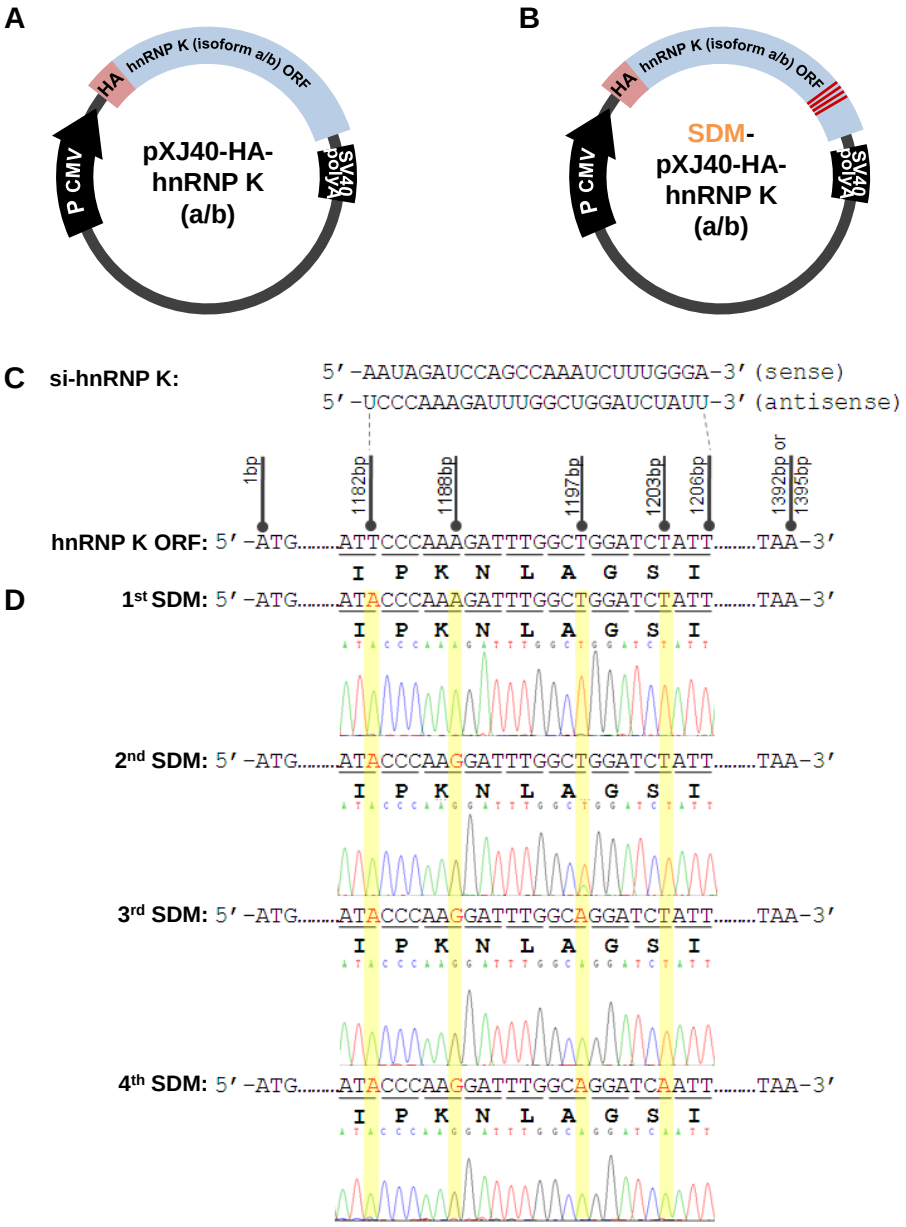


**Figure 3.16 Generation of the SDM-pXJ40-HA-hnRNP K (isoform–a/b) constructs**

**(A)** Plasmid map of pXJ40-HA-hnRNP K–a or –b. hnRNP K–a and –b were amplified from hnRNP K isoform–a and –b expression vectors cloned in previous study (Ng et al., 2005) and sub-cloned into pXJ40-HA vector to form pXJ40-HA-hnRNP K–a or –b. **(B)** Plasmid map of SDM-pXJ40-HA-hnRNP K–a or –b. With the use of pXJ40-HA-hnRNP K–a or –b as template in site-directed mutagenesis, four silent mutations (in red) were introduced to generate SDM- pXJ40-HA-hnRNP K–a or –b to prevent si-hnRNP K from targeting the expression vectors for degradation. **(C)** The sequence of si-hnRNP K and the region on the ORF of hnRNP K that the siRNA targets. **(D)** DNA chromatographs of hnRNP K expression plasmids showing the nucleotide changed (in red) in each successive site-directed mutagenesis of the four selected nucleotides for silent mutations (highlighted in yellow). The 4$^{th}$ SDM reaction yields SDM-pXJ40-HA-hnRNP K–a or –b.

To evaluate if the expression plasmids were able to express hnRNP K when co-transfected with si-hnRNP K, Hep 3B cells were transfected with either 20 nM of non-specific control siRNA (si-ctrl) or si-hnRNP K together with 2 μg of either expression plasmids or empty vector control. Total hnRNP K levels (endogenous and exogenously introduced) as well as exogenously introduced hnRNP K levels were examined using western blot analyses with the use of antibody-specific against hnRNP K and HA-tag respectively (Figure 3.17).

From Figure 3.17B, it could be observed that si-hnRNP K was able to reduce levels of the endogenous protein as previously shown in Figure 3.8B. As si-hnRNP K was able to target pXJ40-HA-hnRNP K –a and –b for degradation, reactions with the co-transfection of si-hnRNP K and these two plasmids had no protein detected with the antibody specific for HA tag (Figure 3.17C and D). In addition, the levels of endogenous hnRNP K were also observed to be reduced in these two reactions.

In contrast, plasmids SDM-pXJ40-HA-hnRNP K–a and –b that carried the four silent mutations were not targeted by si-hnRNP K for degradation and had stable protein expression after 24 h of transfection as detected by antibody against HA-tag (Figure 3.17E and F). As the antibody specific against hnRNP K detects both endogenous and exogenously introduced hnRNP K, it could be observed that the protein levels were no longer reduced in reactions where they were attempted to be restored.

In all, the four silent mutations introduced into expression plasmids pXJ40-HA-hnRNP K–a and –b were able to prevent the plasmids from being targeted by si-hnRNP K for degradation. These plasmids were hence employed in subsequent investigations of apoptosis resistance by hnRNP K.

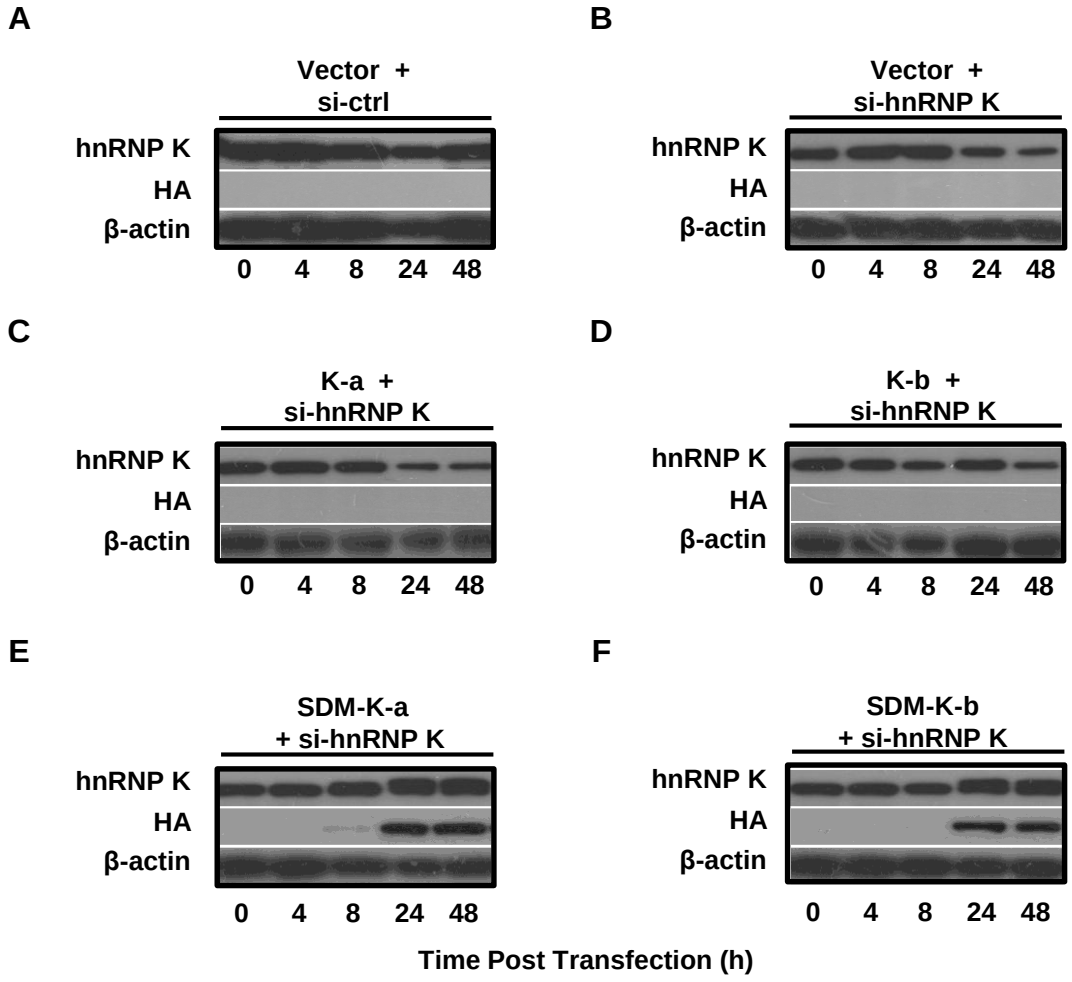


**Figure 3.17 hnRNP K expression constructs with four silent mutations escape degradation induced by si-hnRNP K**

The ability of si-hnRNP K to target SDM-pXJ40-HA-hnRNP K–a or –b for degradation was tested. Western blot analyses examining total hnRNP K and exogenously introduced hnRNP K (HA-tagged) protein expression at various time points indicated in Hep 3B cells co-transfected with **(A)** 2 μg pXJ40HA empty vector and 20 nM si-ctrl, **(B)** 2 μg pXJ40HA empty vector and 20 nM si-hnRNP K, **(C)** 2 μg pXJ40HA-hnRNP K-a (abbreviated as K-a) and 20 nM si-hnRNP K, **(D)** 2 μg pXJ40HA-hnRNP K-b (abbreviated as K-b) and 20 nM si-hnRNP K, **(E)** 2 μg SDM-pXJ40HA-hnRNP K-a (abbreviated as SDM-K-a)and 20 nM si-hnRNP K, **(F)** 2 μg SDM-pXJ40HA-hnRNP K-b (abbreviated as SDM-K-b) and 20 nM si-hnRNP K. β-actin protein level that served as loading control was also examined.

### 3.6.2 Restoration of hnRNP K levels reduces apoptosis

Having demonstrated that the introduction of four silent mutations in the hnRNP K expression constructs could prevent si-hnRNP K mediated degradation, transfection treatments were formulated (Table 3.2) to investigate hnRNP K's specificity and anti-apoptotic property in apoptosis induction resulting from its downregulation.

**Table 3.2 Transfection combinations for the investigation on hnRNP K's specificity and anti-apoptotic property**

| Transfection Treatments | siRNA (20 nM) | Expression construct (2 μg) |
|---|---|---|
| Control | si-ctrl | pXJ40-HA-vector |
| hnRNP K Downregulation | si-hnRNP K | pXJ40-HA-vector |
| Rescue hnRNP K–a | si-hnRNP K | SDM-pXJ40-HA-hnRNP K–a |
| Rescue hnRNP K–b | si-hnRNP K | SDM-pXJ40-HA-hnRNP K–b |

With the formulated transfection combinations, Hep 3B cells were transfected and subjected to TUNEL assay analysis as shown in Figure 3.18A. Consistent with the results presented earlier (Figure 3.12B), increase in percentage of apoptotic cells could be observed in cells with hnRNP K reduced as compared to control, as exemplified by the increase in percentage of TUNEL positive cells in the total cell population sampled (Figure 3.18B). It was also observed that with the restoration of hnRNP K levels, the percentage of TUNEL positive cells in the total cell population sampled was reduced. hnRNP K downregulation treatment had 40.8±6.5% of TUNEL positive cells while rescue hnRNP K–a and –b treatments had 23.58±2.6% ($p<0.01$) and 30.7±0.54% ($p<0.05$) of TUNEL positive cells respectively. The results have thus indicated that the apoptosis induced was indeed hnRNP K-specific and suggested that both isoforms of hnRNP K possess anti-apoptotic property.

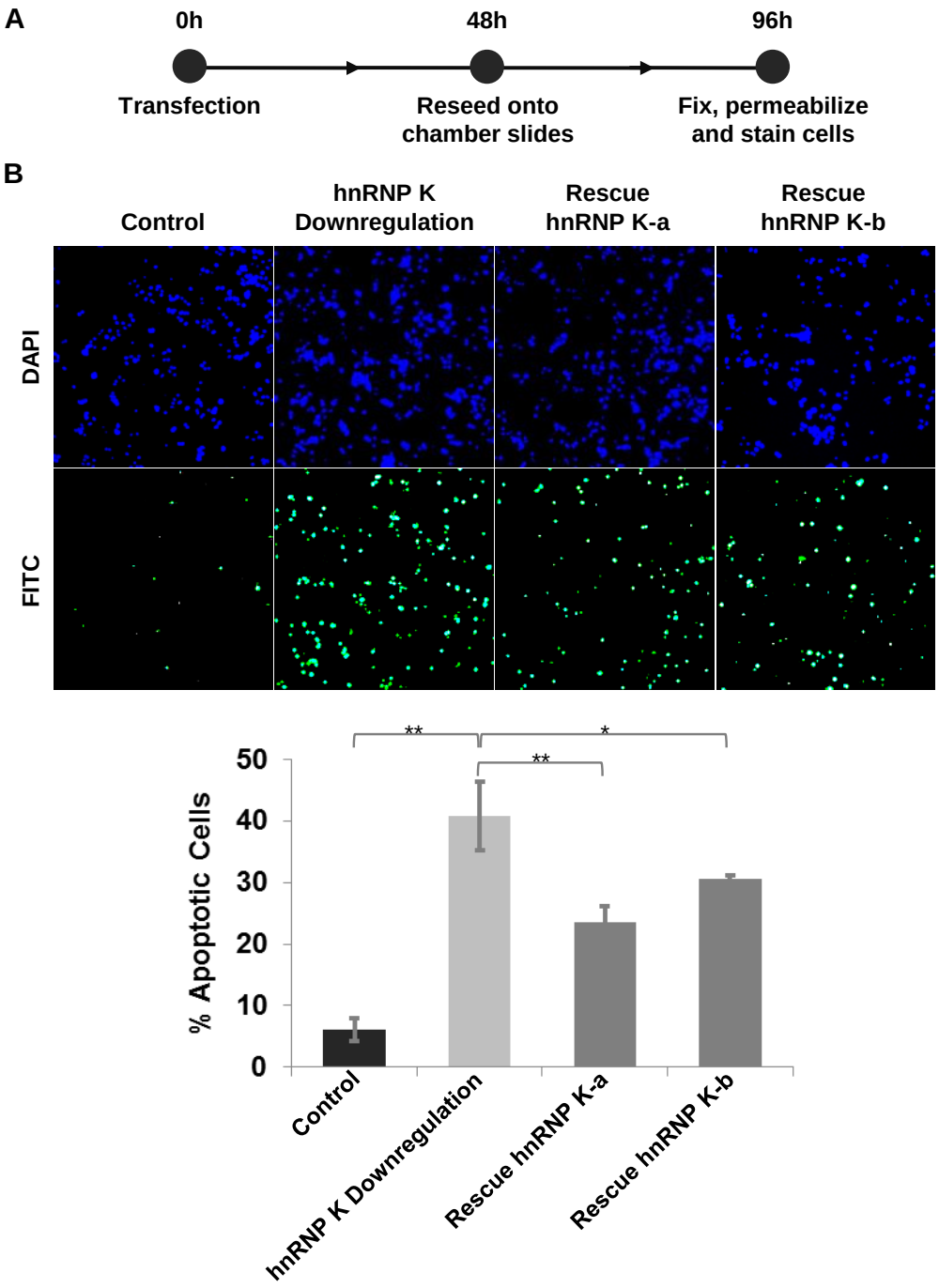


**Figure 3.18 Restoring hnRNP K levels reduces the percentage of TUNEL positive cells**

**(A)** TUNEL assay experimental procedure, where Hep 3B cells were reseeded into chamber slides 48 h post-transfection and cells were fixed, permeabilised, labelled before mounting with mounting agent containing DAPI. **(B)** Differentially transfected cells were observed under fluorescence microscope, where DAPI-stained and FITC-stained cells represented total cell population and cells undergoing apoptosis respectively. DAPI-stained and FITC-stained cells for each slide were counted with Image-Pro software and data were expressed as the mean ± SD % of FITC-stained cells of the total cell population (DAPI-stained) obtained from triplicates. (**p<0.05*, ***p<0.01*). Results from a representative experiment of at least two with similar trend.

To further examine whether the restoration of hnRNP K levels could reduce the extent of induced apoptosis following its reduction, Hep 3B cells were differentially transfected with the various transfection combinations listed in Table 3.2, fixed, stained with propidium iodide and subjected to subsequent cell cycle analysis, where hypodiploid cells (sub-$G_0/G_1$ peak) were quantified with fluorescence-activated cell sorter (FACS).

From the FACS quantification data depicted in Figure 3.19, it could be observed that hnRNP K reduction resulted in an increase in percentage of hypodiploid cells as compared to control at 72 h post-transfection. This was consistent with the results presented earlier that indicated the induction of apoptosis following hnRNP K downregulation.

In addition, the percentage of hypodiploid cells for both rescue hnRNP K–a and –b treatments were lower as compared to hnRNP K knockdown treatment. The percentage of hypodiploid cells in rescue hnRNP K–a and –b treatments was 15.3% and 18.6% respectively while that in cells with hnRNP K downregulated was 24.6%. Taken together with the findings from the TUNEL assay, the results have indicated that the extent of apoptosis induced by hnRNP K reduction was significantly reduced following the attempt to restore its expression levels.

Interestingly, by comparing the data from the TUNEL assay (Figure 3.18B) and the cell cycle analysis (Figure 3.19), it was observed that the restoration of hnRNP K isoform–a was able to reduce the extent of apoptosis induced slightly better than isoform–b. As depicted in Figure 3.18B, percentage of TUNEL positive cells in rescue hnRNP K–a treatment was slightly lesser than in rescue hnRNP K–b treatment (23.6±2.6% versus 30.7±0.6%). Moreover, as revealed by cell cycle analysis, the percentage of hypodiploid cells for rescue hnRNP K–a treatment was lower as

compared to hnRNP K–b treatment (15.3% versus 18.6%). This was likely to be due to the slight increase in the expression level or stability of hnRNP K isoform–a as compared to isoform–b (Figure 3.17B), which resulted in a slight decrease in percentage of apoptotic cells in rescue hnRNP K–a as compared to hnRNP K–b treatment. Nonetheless, restoration of hnRNP K expression levels with either isoform was able to reduce apoptosis that was induced by hnRNP K downregulation, thus suggesting that the observed apoptotic effect was indeed hnRNP K-specific and also revealed that hnRNP K possesses anti-apoptotic property that deserves further investigation.

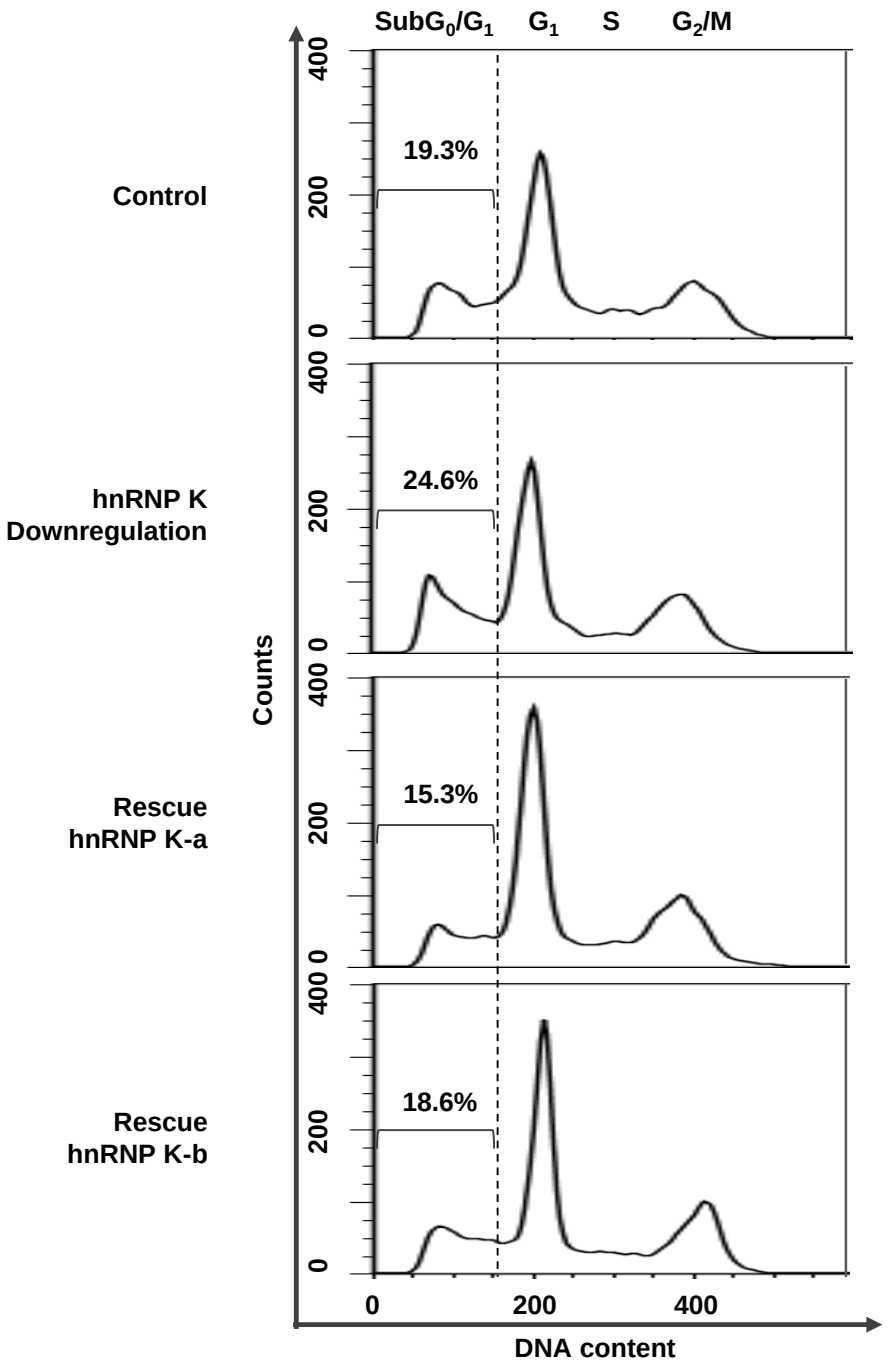


**Figure 3.19 Restoration of hnRNP K expression levels decreases the percentage of sub$G_0/G_1$ cells**

Cell cycle profiles of the differentially transfected Hep 3B cells (according to Table 3.2) and their percentage of hypodiploid cells (sub$G_0/G_1$) for each transfection reaction at both 72 h post-transfection were indicated. Results from a representative experiment of at least two with similar trend.

Taken together, the various functional assays performed in this current study (Table 3.3) have established that the downregulation of hnRNP K indeed induces apoptosis. As observed with WST-1 assay and FACS quantification of hypodiploid cells, loss of cell viability and increase in cell death were observed following the reduction of hnRNP K. Furthermore, the detection of DNA fragmentation observed in apoptosis with the use of TUNEL assay, coupled with the detection of apoptosis-specific events such as activation of caspase-3/7 activity and production of caspase-cleaved 89kDa PARP-1 fragment all pointed towards the specific induction of apoptosis. The data has also revealed that extended period of hnRNP K reduction induces apoptosis, specifically only from 72 h post-transfection onwards, regardless of whether HCC cells were transfected with 20 nM or 40 nM of hnRNP K-specific siRNA. Taken together with the observation where restoration of hnRNP K expression levels reduces the extent of apoptosis induced, the results have indicated that the downregulation of hnRNP K most likely caused the cells to lose their ability to inhibit apoptosis and suggested that hnRNP K exerts an anti-apoptotic effect in HCC cells.

**Table 3.3 Summary of results for functional assays investigating the effect of hnRNP K's downregulation**

| | | Current Study (With decreased hnRNP K) | |
|---|---|---|---|
| **Functional Assays** | **Expected Outcomes in different types of Programmed Cell Death*** | **Results** | **Type of Cell death*** |
| WST-1 cell viability assay | Does not distinguish between apoptosis and necrosis, only indicates cell viability (Galluzzi et al., 2009) | Reduced cell viability | Inconclusive |
| Immunoblot detection of 89kDa cleaved PARP-1 | Found only in apoptotic cells, other cleavage pattern present in necrotic cells (Gobeil et al., 2001; Soldani and Scovassi, 2002) | Level increased | Apoptosis |
| TUNEL assay | Gold standard to detect apoptosis *in situ*, but false positive may result from necrotic death in some cases such as due to sample processing (Galluzzi et al., 2009) | TUNEL positive cells increased | Apoptosis |
| Caspase-3/7 activity | Caspase activation found only in apoptotic and not in necrotic cells; but caspase activation may occur in cell death-unrelated scenarios (Galluzzi et al., 2009) | Caspase-3/7 activity increased | Apoptosis |
| FACS quantification of hypodiploid cells-(sub-$G_0/G_1$ peak) | Cell death monitored by the quantification of events with DNA content lesser than diploid (Kroemer et al., 2009); not only specific for apoptosis (Lecoeur, 2002). | Percentage of hypodiploid cells increased | Inconclusive |

*Types of programmed cell death referred to either apoptosis or necrosis.

## 3.7 Chapter Summary

With the use of various HCC cell lines, hnRNP K has been demonstrated to be abundant at the protein level. Similar to several other chemotherapeutic drugs that can reduce the levels of hnRNP K as well as result in cell death (Barboro et al., 2009; Benelli et al., 2009; Enge et al., 2009; Rahman-Roblick et al., 2007), 5-FU that has been utilised for HCC treatment can downregulate hnRNP K independently of p53 at the mRNA level. As 5-FU is able to reduce the viability of HCC cells and the levels of hnRNP K concurrently, it is proposed that the reduction of hnRNP K is likely one of the mechanisms for 5-FU to cause cell death observed in HCC cell lines. Indeed, downregulation of hnRNP K solely with specific siRNA reduced cell viability and changed the morphology of HCC cells. Further functional analyses provided evidence for the onset of apoptosis brought about by hnRNP K reduction and that this phenomenon observed was indeed hnRNP K-specific. Moreover, it was also shown that the apoptosis induced by the reduction of hnRNP K was p53-independent. Current data also indicated that hnRNP K plays an anti-apoptotic role, for its downregulation only induces apoptosis specifically at 72 h post-transfection even with different amount of siRNA specific against hnRNP K. These have indicated that hnRNP K downregulation compromises the HCC cells' ability to inhibit apoptosis, which warrants further investigation.

# CHAPTER 4

## The Anti-apoptotic Mechanism of hnRNP K

## 4.1 Introduction

In Chapter 3, it was demonstrated that the reduction of hnRNP K induces apoptosis in HCC cell lines. In this chapter, the mechanism behind hnRNP K's anti-apoptotic effect was further examined.

Of the various studies that have shown that the downregulation of hnRNP K induced cell death, few have attempted to dissect the mechanism behind this phenomenon. Evidence for the involvement of the intrinsic pathway was shown by White et al. (2010), where the downregulation of hnRNP K in Sy5y neuroblastoma cell line induced apoptosis that was accompanied by the translocation of PKCδ to the mitochondria. Conversely, Chen et al. (2010) demonstrated the sensitization of nasopharyngeal carcinoma (NPC) cells to TRAIL-induced apoptosis after 48 h of hnRNP K downregulation, which the authors attributed to the downregulation of cFLIP, a downstream transcriptional target of hnRNP K. This indicated that possible involvement of the extrinsic apoptotic pathway. Hence, the exact anti-apoptotic property that hnRNP K confers is still unclear, particularly in HCC cells. Therefore, this chapter aims to elucidate the anti-apoptotic mechanism of hnRNP K with the use of HCC cell lines.

Given that caspases are well-known executors of apoptosis and that caspase-3/7 activity correlates positively with apoptosis induced by hnRNP K downregulation (as shown in Chapter 3), the anti-apoptotic mechanism of hnRNP K was studied primarily with the measurement of caspase-3/7 activity. In addition, as hnRNP K downregulation in Hep 3B cells had revealed a significant decrease of its nuclear expression (Figure 3.2), its role as a transcription factor was also briefly explored in an attempt to elucidate its anti-apoptotic function.

## 4.2 Caspase-8 is essential for downstream caspase-3/7 activation in apoptosis induced by hnRNP K reduction

As suggested by previous studies, both the extrinsic and intrinsic apoptotic pathways could possibly contribute to the apoptosis induction observed following hnRNP K reduction. Hence, to examine the contribution of the extrinsic and intrinsic apoptotic pathway, as represented by caspase-8 and caspase-9 respectively, to the apoptosis induced in HCC cells by hnRNP K downregulation, the two caspases were individually silenced simultaneously with hnRNP K using siRNAs in Hep 3B and HepG2 cells. The activity of caspase-3/7 was subsequently measured at 72 h post-transfection, a time point where apoptosis induction had been repeatedly detected as shown in the previous chapter.

The results intriguingly revealed a dramatic decrease in caspase-3/7 activity when caspase-8 was silenced together with hnRNP K (Figure 4.1A). As compared to cells with only hnRNP K downregulated, the caspase-3/7 activity observed in cells with co-knockdown of caspase-8 and hnRNP K decreased by 71.6% ($p<0.01$) and 90.6% ($p<0.01$) in Hep 3B and HepG2 respectively. This indicated that caspase-8 is crucial in facilitating hnRNP K downregulation-induced apoptosis at 72 h post-transfection, as the knockdown of caspase-8 resulted in a strong inhibition of caspase-3/7 activation.

In contrast, the co-knockdown of caspase-9 and hnRNP K resulted in an increase in caspase-3/7 activity. As compared to cells with solely hnRNP K downregulated, the caspase-3/7 activity observed in cells with co-knockdown of caspase-9 and hnRNP K increased by 51.0% ($p<0.01$) and 124.1% ($p<0.01$) in Hep 3B and HepG2 respectively. Unlike caspase-8, the knockdown of caspase-9 failed to reduce caspase-3/7 activation induced by hnRNP K downregulation and instead intensified the activation. The reason behind this enhancement is currently unknown, but the results

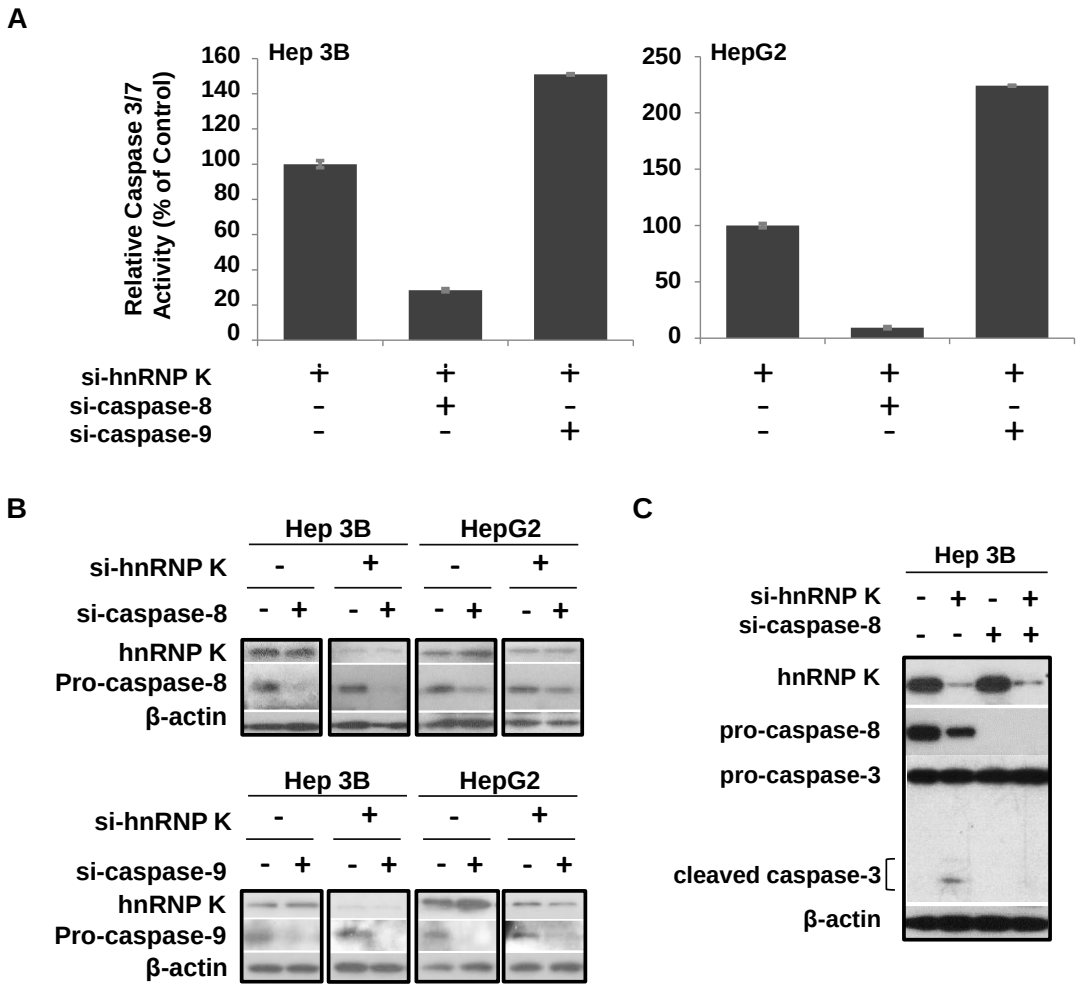


**Figure 4.1 Silencing caspase-8 prevents caspase-3/7 activation following hnRNP K reduction**

**(A)** Caspase-3/7 activity in differentially transfected Hep 3B and HepG2 cells was measured at 72 h post-transfection. Cells were transfected with 20 nM of si-hnRNP K with or without 20 nM of si-caspase-8 or si-caspase-9 as indicated. Data was expressed as relative caspase-3/7 activity of that transfected with si-hnRNP K and presented as mean ± SD % of triplicates. Results are from a representative experiment of at least two with similar trend. **(B)** Reduction of the respective caspases by their specific siRNAs at 48 h post-transfection was verified with western blot and β-actin protein level that served as loading control was also examined. **(C)** Protein levels of pro-caspase-3 as well as activated and cleaved caspase-3 were also examined in differentially transfected Hep 3B at 72 h post-transfection.

have clearly indicated that caspase-8 apoptotic signalling is essential for downstream caspase-3/7 activation observed during hnRNP K downregulation.

Reduction of the respective caspases by their specific siRNAs was also verified at the protein level (Figure 4.1B). When levels of caspase-3 were examined in Hep 3B, activated and cleaved forms were detected only in cells with hnRNP K knocked-down but not in cells with caspase-8 simultaneously downregulated. Moreover, the levels of pro-caspase-3 were comparable across the differentially transfected cells, indicating that the observed reduction in caspase-3/7 activity following co-knockdown of hnRNP K and caspase-8 was not due to a reduction of effector caspases expression levels. These strongly suggest that hnRNP K exerts its anti-apoptotic effect on the caspase-8 apoptotic signalling pathway.

## 4.3 hnRNP K downregulation amplifies the apoptotic effect of TRAIL

### 4.3.1 Intact TRAIL-mediated apoptotic signalling in HCC cell lines

Having demonstrated that hnRNP K exerts its anti-apoptotic effect mainly via caspase-8 signalling and that apoptosis occurs spontaneously only after 72 h of hnRNP K downregulation (Figure 3.13), it was further examined whether the anti-apoptotic effect of hnRNP K could have been affected at earlier time points of hnRNP K downregulation i.e. 24 h post-transfection.

To investigate this possibility, caspase-8 apoptotic signalling pathway that is best represented by the extrinsic apoptotic pathway was induced using death ligands after 24 h of hnRNP K downregulation and cells were examined for any amplification in death signals induced. As FAS-ligand (FASL) and TRAIL are capable of inducing apoptosis in hepatocytes (Malhi et al., 2010), their ability to induce apoptosis in HCC

cells was first tested, by treating Hep 3B and HepG2 cells with increasing concentrations of each ligand before subjecting them to Caspase-Glo® 3/7 Assay.

As shown in Figure 4.2, with increasing amount of FASL from 2-100 ng/ml, there was an increase in the activity of caspase-3/7 in HepG2, over an incubation period of 2 h to 8 h. Activity of caspase-3/7 was the strongest with 8 h of 100 ng/ml FASL treatment in HepG2, with a normalised caspase-3/7 activity of 175.6±4.0% as compared to untreated control. On the contrary, caspase-3/7 activity remained at its basal state in Hep 3B even with increasing concentrations of FASL. This observation for Hep 3B was consistent with a previous report that the resistance of Hep 3B to FAS-mediated apoptosis is due to a lack of surface expression of functional FAS (receptor) (Lamboley et al., 2002).

In contrast to FASL, TRAIL as shown in Figure 4.3, was able to increase caspase-3/7 activity in both Hep 3B and HepG2 with increasing concentrations of 2-50 ng/ml over the incubation period of 2 h to 8 h. Specifically in HepG2, the increase in caspase-3/7 activity correlated with both the increase in TRAIL concentration as well as treatment time. However, it could be observed that the caspase-3/7 activity in Hep 3B peaked at 4h post-TRAIL treatment and thereafter declined slightly. Nonetheless, comparing the effect of TRAIL with FASL in the induction of apoptosis, as exemplified by the increase in caspase-3/7 activity, TRAIL but not FASL was able to increase caspase-3/7 activity in Hep 3B. Taken together, these suggested that signalling pathway in HepG2 and Hep 3B for TRAIL-mediated apoptosis were functional and TRAIL was hence utilised as a death ligand for the induction of extrinsic apoptotic pathway for downstream analyses.

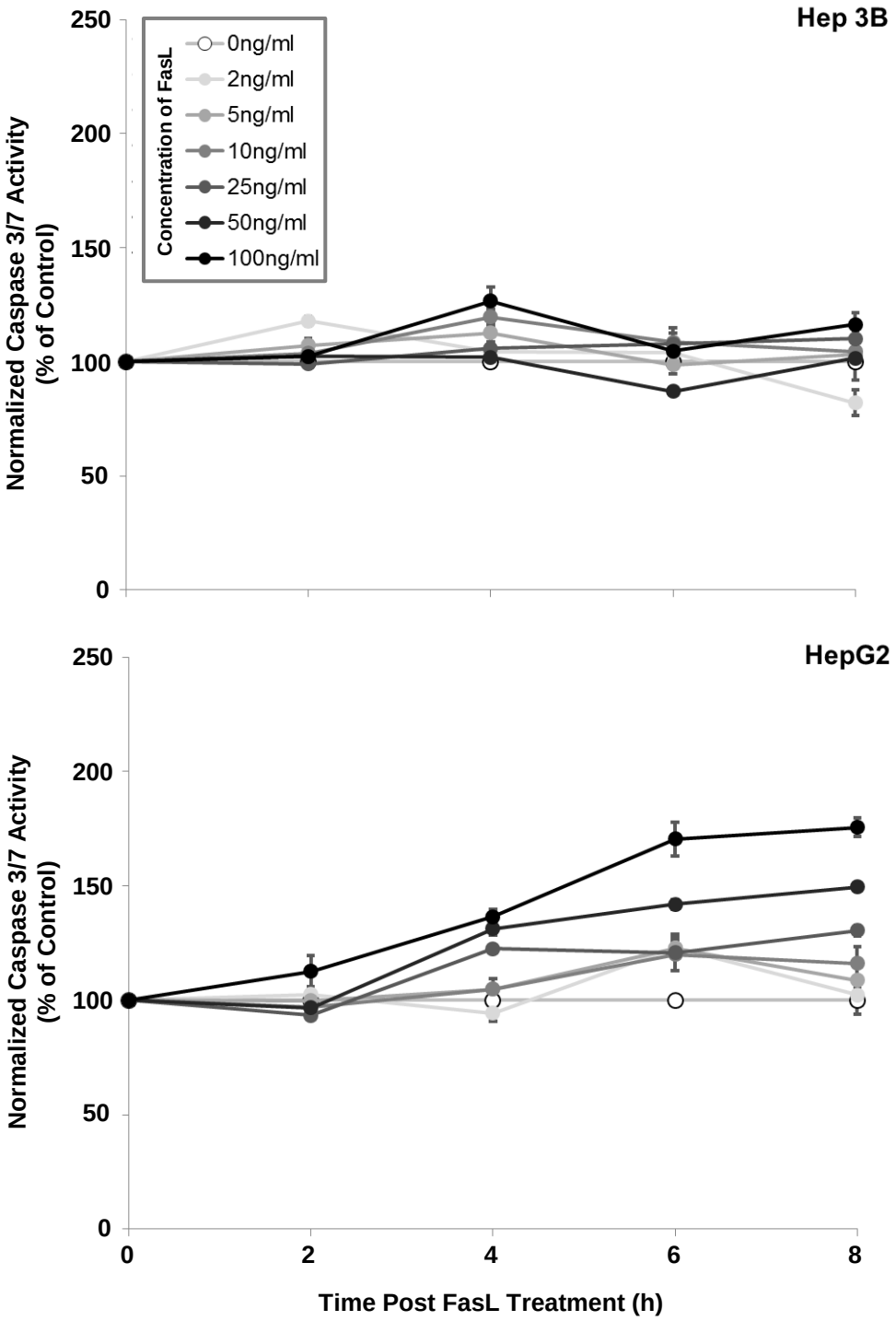


**Figure 4.2 Hep 3B is resistant to apoptosis induced by FASL**

The ability of FASL in activating caspase-3/7 in Hep 3B and HepG2 was examined. Hep 3B (upper panel) and HepG2 (lower panel) cells were treated with different concentrations (0-100 ng/ml) of FASL over an incubation period of 2-8 h as indicated before caspase-3/7 activity was measured with Caspase-Glo® 3/7 assay. Caspase-3/7 activity was expressed as caspase-3/7 activity of each differentially treated cell normalised to that in cells untreated and data were presented as mean ± SD% of triplicates.

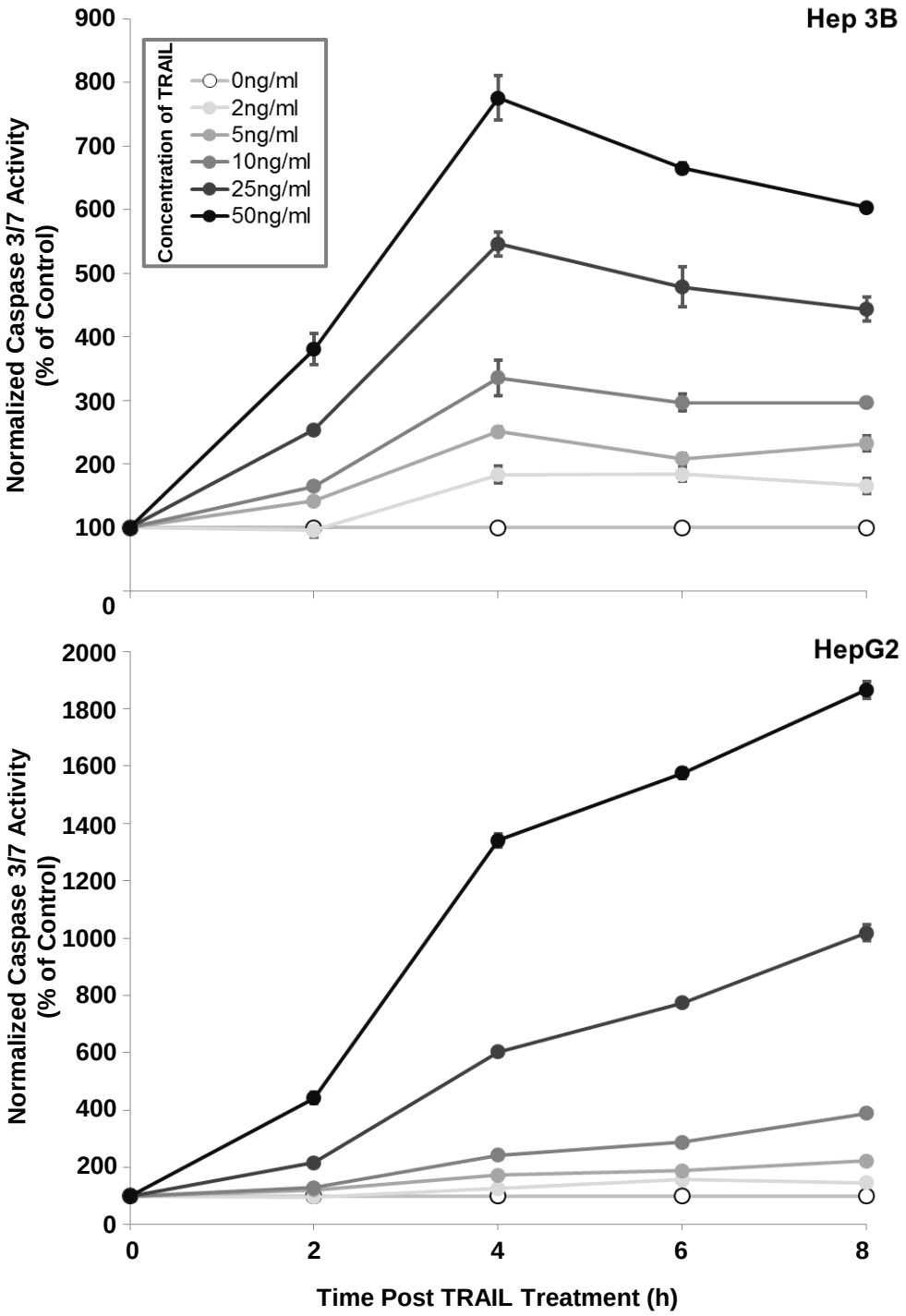


**Figure 4.3 TRAIL signalling is intact in Hep 3B and HepG2**
The ability of TRAIL in activating caspase-3/7 in Hep 3B and HepG2 was examined. Hep 3B (upper panel) and HepG2 (lower panel) cells were treated with different concentrations (0-50 ng/ml) of TRAIL over an incubation period of 2-8 h as indicated before caspase-3/7 activity was measured with Caspase-Glo® 3/7 assay. Data was expressed as caspase-3/7 activity of each differentially treated cell normalised to that in cells untreated at each time point and presented as mean ± SD% of triplicates.

### 4.3.2 hnRNP K downregulation sensitises HCC cells to TRAIL-mediated apoptotic signals

TRAIL has been demonstrated to induce apoptosis mainly via the extrinsic pathway upon its binding to DR4 or DR5 as introduced in Section 1.2.3 and its signalling pathway was also revealed to be intact in both Hep 3B and HepG2 cells (Figure 4.3). Hence, to examine whether the anti-apoptotic effect of hnRNP K could have been affected at earlier time point of hnRNP K downregulation, where apoptosis induction was not detected, Hep 3B and HepG2 with hnRNP K reduced for 24 h were treated with low dose of TRAIL (2 ng/ml) before their caspase-3/7 activity was determined (Figure 4.4A).

From Figure 4.4, it could be observed that the reduction of hnRNP K resulted in an enhancement of caspase-3/7 activity in TRAIL-treated Hep 3B and HepG2 cells. Firstly, in both cell lines without TRAIL treatment, no significant difference in caspase-3/7 activity was observed between cells with or without hnRNP K downregulated at the various time points tested. This was consistent with the results demonstrated in Chapter 3, where earlier time points of hnRNP K reduction did not result in the onset of apoptosis.

Next, comparing the caspase-3/7 activity in differentially transfected Hep 3B cells treated with 2 ng/ml TRAIL, there was an increase in caspase-3/7 activity of 45.4% and 174.3% following 2 h and 8 h of TRAIL treatment respectively in hnRNP K downregulated cells as compared to control (Figure 4.4B). Moreover, in HepG2, the increment in caspase-3/7 activity was higher, where an increase in caspase-3/7 activity of 471.3% and 541.7% following 2 h and 8 h of TRAIL treatment respectively was observed in hnRNP K downregulated cells as compared to control (Figure 4.4C). Thus, the results revealed that even though hnRNP K reduction at early

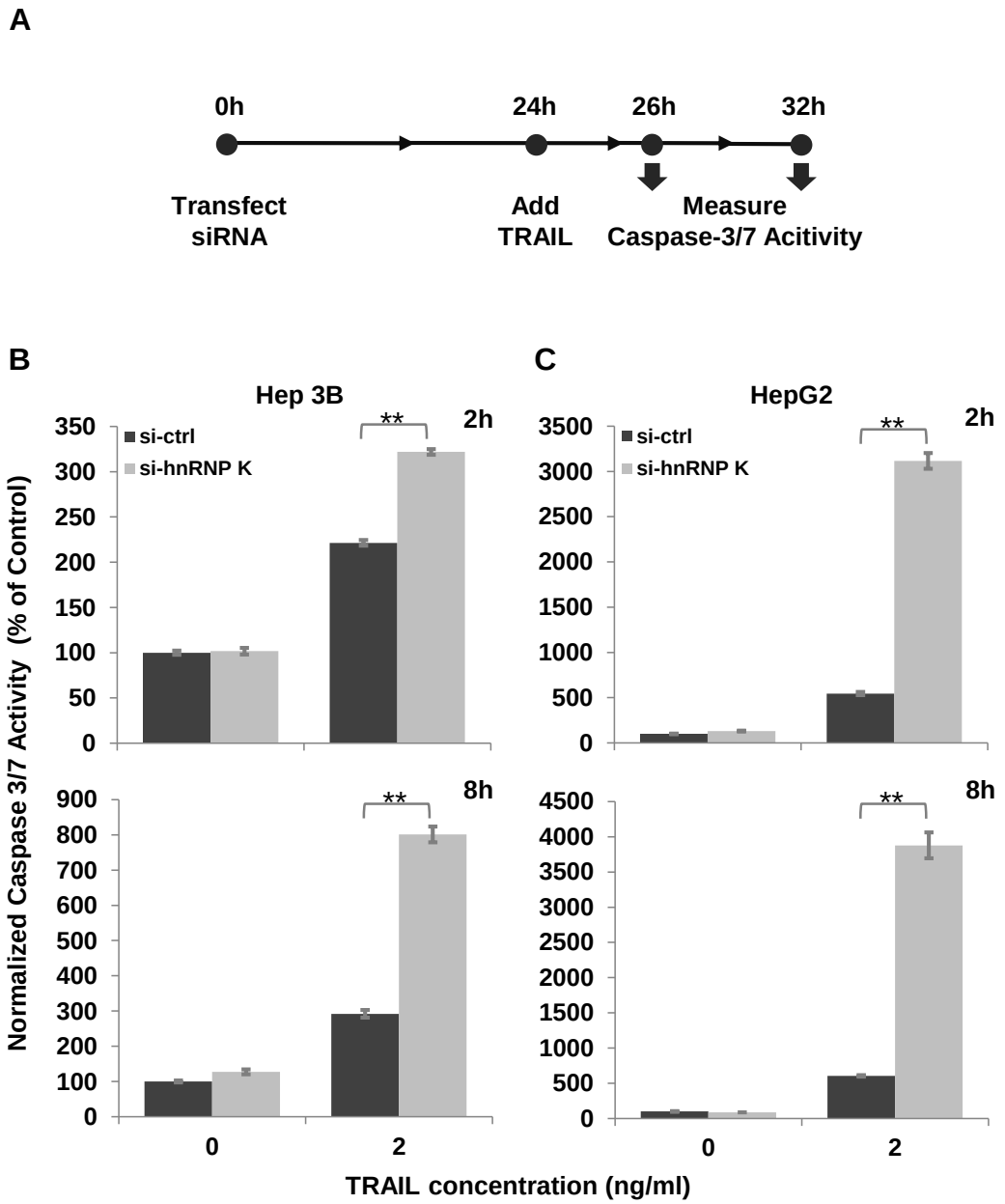


**Figure 4.4 hnRNP K downregulation enhances TRAIL-induced caspase-3/7 activity**

**(A**) Experimental workflow to examine the anti-apoptotic function of hnRNP K with TRAIL. Hep 3B and HepG2 cells were transfected with 40 nM of either si-ctrl or si-hnRNP K for 24 h before the addition of 2 ng/ml of TRAIL. Cells were collected following 2 h and 8 h of incubation with TRAIL before the measurement of caspase-3/7 activity. Caspase-3/7 activity of differentially transfected and treated **(B)** Hep 3B and **(C)** HepG2 was shown and data was expressed as caspase-3/7 activity of each differentially transfected and treated cells normalised to that in cells transfected with si-ctrl and untreated with TRAIL at each time point and presented as mean ± SD% of triplicates. (**$p$<0.01). Results are from a representative experiment of at least two with similar results trend.

time points did not induce apoptosis, it was able to sensitise cells to TRAIL-induced apoptosis.

Having demonstrated that hnRNP K downregulation augmented caspase-3/7 activity in TRAIL-treated cells, it was subsequently examined whether this effect was hnRNP K-specific. Thus, increasing concentrations of si-hnRNP K were transfected into Hep 3B and HepG2 cells to generate a gradient of hnRNP K knockdown. Following 24 h of transfection, the cells were treated with low dose of TRAIL before caspase-3/7 activity measurement (Figure 4.5A).

As reflected from Figure 4.5B, the enhancement in TRAIL-induced caspase-3/7 activity increased in both Hep 3B and HepG2 with greater hnRNP K downregulation using increasing concentrations of si-hnRNP K from 0 nM to 20 nM. Caspase-3/7 activity in TRAIL-treated cells that were pre-transfected with 10 nM and 20 nM of si-hnRNP K increased by 82.9% and 150.2% respectively in Hep 3B ($p<0.01$) and by 960% and 1308.7% in HepG2 ($p<0.01$) as compared to control.

However, when the concentration of siRNA increased from 20 nM to 40 nM, augmented TRAIL-induced caspase-3/7 activity was observed to decrease instead. A plausible explanation could be that the enhanced caspase-3/7 activity brought about by 40 nM of si-hnRNP K had attained its peak at earlier time points of TRAIL treatment and had subsequently started to decline. In addition, it was also observed that there was a huge percentage difference in the enhancement of TRAIL-induced caspase-3/7 activity between both cell lines following hnRNP K downregulation. Though this disparity is unclear, it could be a result of cell line difference.

Nonetheless, the trend in enhancement of TRAIL-induced caspase-3/7 following hnRNP K reduction was similar in both cell lines and this indicated that the observed

enhancement of caspase-3/7 activity in TRAIL-treated Hep 3B and HepG2 was indeed hnRNP K-specific.

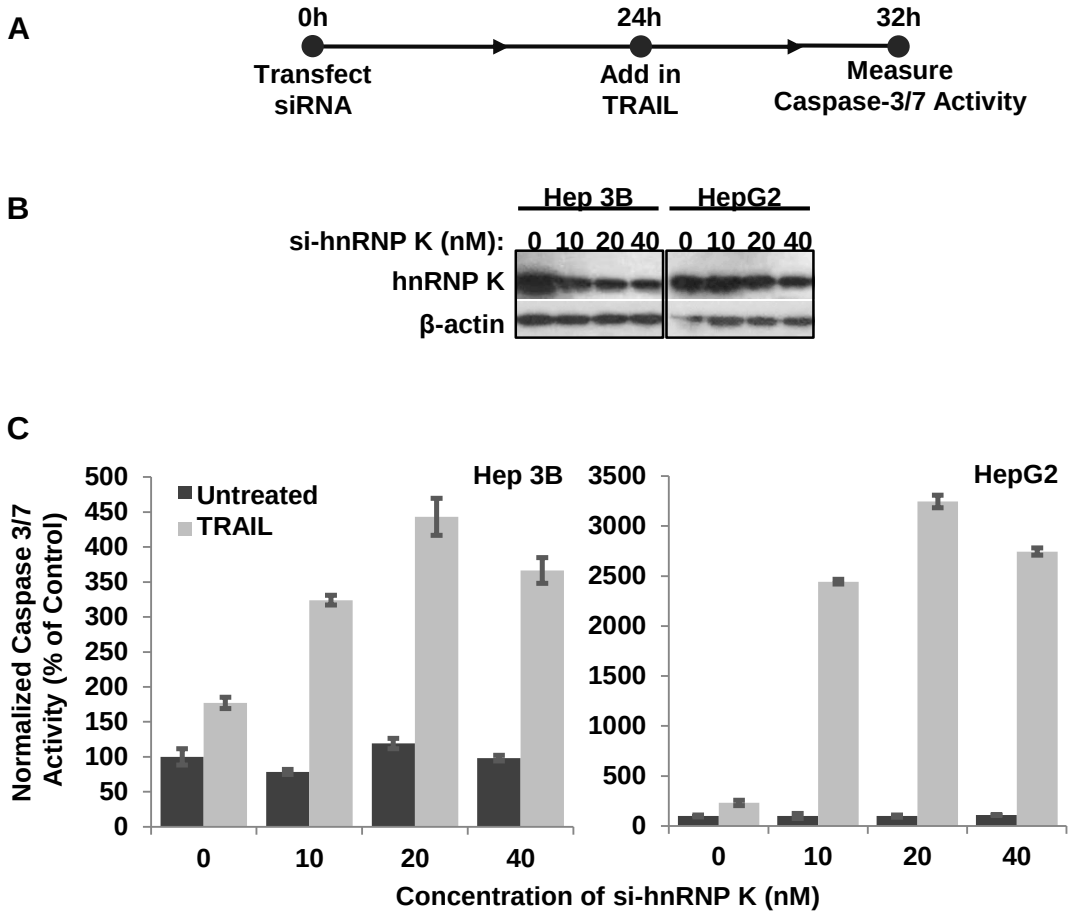


**Figure 4.5 Enhancement of TRAIL-induced caspase-3/7 activity depends on hnRNP K**

hnRNP K's specificity in enhancing TRAIL-induced caspase-3/7 activity was examined. (**A**) Hep 3B and HepG2 were transfected with increasing concentrations of si-hnRNP K for 24 h before 8 h of incubation with 2 ng/ml of TRAIL and subsequent caspase-3/7 activity measurement. (**B**) Increased knockdown of hnRNP K at the protein level with increased concentration of siRNA was verified in both Hep 3B and HepG2 at 24 h post-transfection. **(C)** Caspase-3/7 activity of the differentially transfected and treated cells were shown. Data was expressed as caspase-3/7 activity of each differentially transfected and treated cells normalised to cells transfected with si-ctrl and untreated with TRAIL at each time point and presented as mean ± SD% of triplicates. Results are from a representative experiment of at least two with similar results trend.

As knocking-down hnRNP K with increasing concentrations of siRNA increased the enhancement in caspase-3/7 activity induced by TRAIL, the direct anti-apoptotic function of hnRNP K on TRAIL-induced caspase-3/7 activation was next examined. For this purpose, the two protein isoforms of hnRNP K were first individually overexpressed in both HCC cell lines and cells were subsequently treated with TRAIL before caspase-3/7 activity measurement (Figure 4.6A).

Results from the caspase-3/7 assay revealed that with the overexpression of hnRNP K regardless of isoform was able to reduce the caspase-3/7 activity induced by TRAIL. As shown in Figure 4.6B, the overexpression of hnRNP K isoform–a and –b reduced TRAIL-induced caspase-3/7 activity when compared to control by 22.4% and 15.0% respectively in Hep 3B and 23.6% and 30.4% respectively in HepG2. This has provided a direct indication of the anti-apoptotic function of hnRNP K on TRAIL-induced apoptosis and was in concordance with the previous observations in Chapter 3 that hnRNP K possesses an anti-apoptotic function.

Together, the data from Figure 4.5 and Figure 4.6 indicates that the levels of hnRNP K negatively correlate with the level of TRAIL-induced caspase-3/7 activity, indicating that hnRNP K likely exerts its anti-apoptotic effect directly or indirectly on the activity of caspases involved in TRAIL-mediated extrinsic apoptotic pathway.

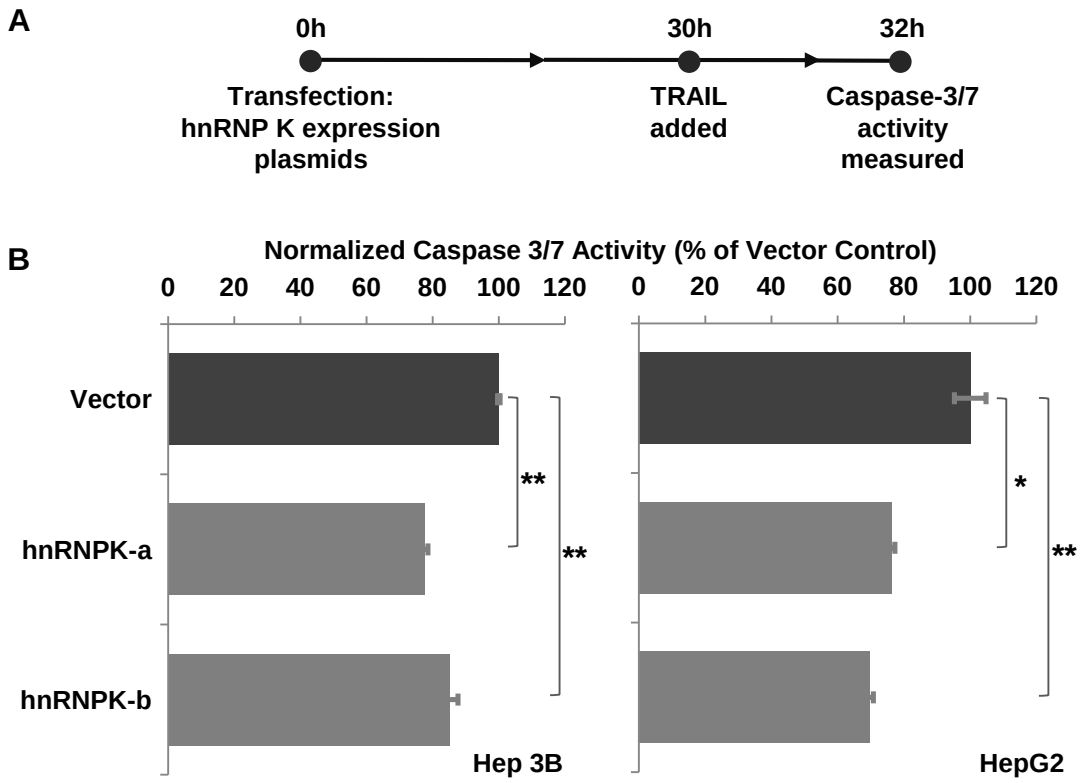


**Figure 4.6 hnRNP K overexpression reduces TRAIL-induced caspase-3/7 activity**
The anti-apoptotic effect of hnRNP K on TRAIL-induced caspase-3/7 activity was examined. **(A**) Hep 3B and HepG2 cells were first transfected with 2 μg of empty pXJ40-HA vector or SDM-pXJ40-HA-hnRNP K (isoform–a or –b) for 30 h before 8 h of incubation with 10 ng/ml of TRAIL and subsequent caspase-3/7 activity measurement. **(B**) Caspase-3/7 activity of the differentially transfected and treated cells were shown. Data was expressed as TRAIL-induced caspase-3/7 activity of each differentially transfected cells normalised empty vector-transfected cells. Results were also presented as mean ± SD% of triplicates from a representative experiment of at least two with similar trend. (**$p$<0.01, *$p$<0.05).

### 4.3.3 Enhanced TRAIL-induced caspase-3/7 activity by hnRNP K downregulation as a result of increased caspase-8 activity

Given that the downregulation of hnRNP K augmented TRAIL-induced caspase-3/7 activation, it was examined whether this effect could have been brought about by enhanced BID cleavage, which could then amplify the apoptotic effect via the intrinsic apoptotic pathway. To investigate this possibility, the profile of TRAIL-induced caspase-3/7 activity was first studied in HepG2 cells that revealed higher enhancement in TRAIL-induced caspase-3/7 activation upon hnRNP K reduction. Thus, hnRNP K was first downregulated in HepG2 and subsequently differentially treated with increasing concentrations of TRAIL (0-50 ng/ml) before caspase-3/7 activity measurement.

As shown in Figure 4.7A, the profiles of TRAIL-induced caspase-3/7 activity in HepG2 cells treated with non-specific control siRNA (si-ctrl) and si-hnRNP K were strikingly different and it was observed that treating si-hnRNP K transfected cells with 2 ng/ml of TRAIL produced similar caspase-3/7 activity as compared to control cells treated with 50 ng/ml of TRAIL (3569.0% as compared to 3610.6%). This indicated that the loss of hnRNP K amplified TRAIL-induced caspase-3/7 activity such that a lower concentration of TRAIL (2 ng/ml) could induce caspase-3/7 activity that was normally achieved only with a high TRAIL concentration (50 ng/ml).

Having known the profiles of TRAIL-induced caspase-3/7 activity in HepG2, BID cleavage induced by TRAIL was examined. Western blot analyses of full length BID in HepG2 cells treated with increasing concentrations of TRAIL revealed increased cleavage of BID as depicted by the decrease in amount of full length BID with increasing TRAIL concentration, which was particularly evident in cells treated with 50 ng/ml of TRAIL (Figure 4.7B).

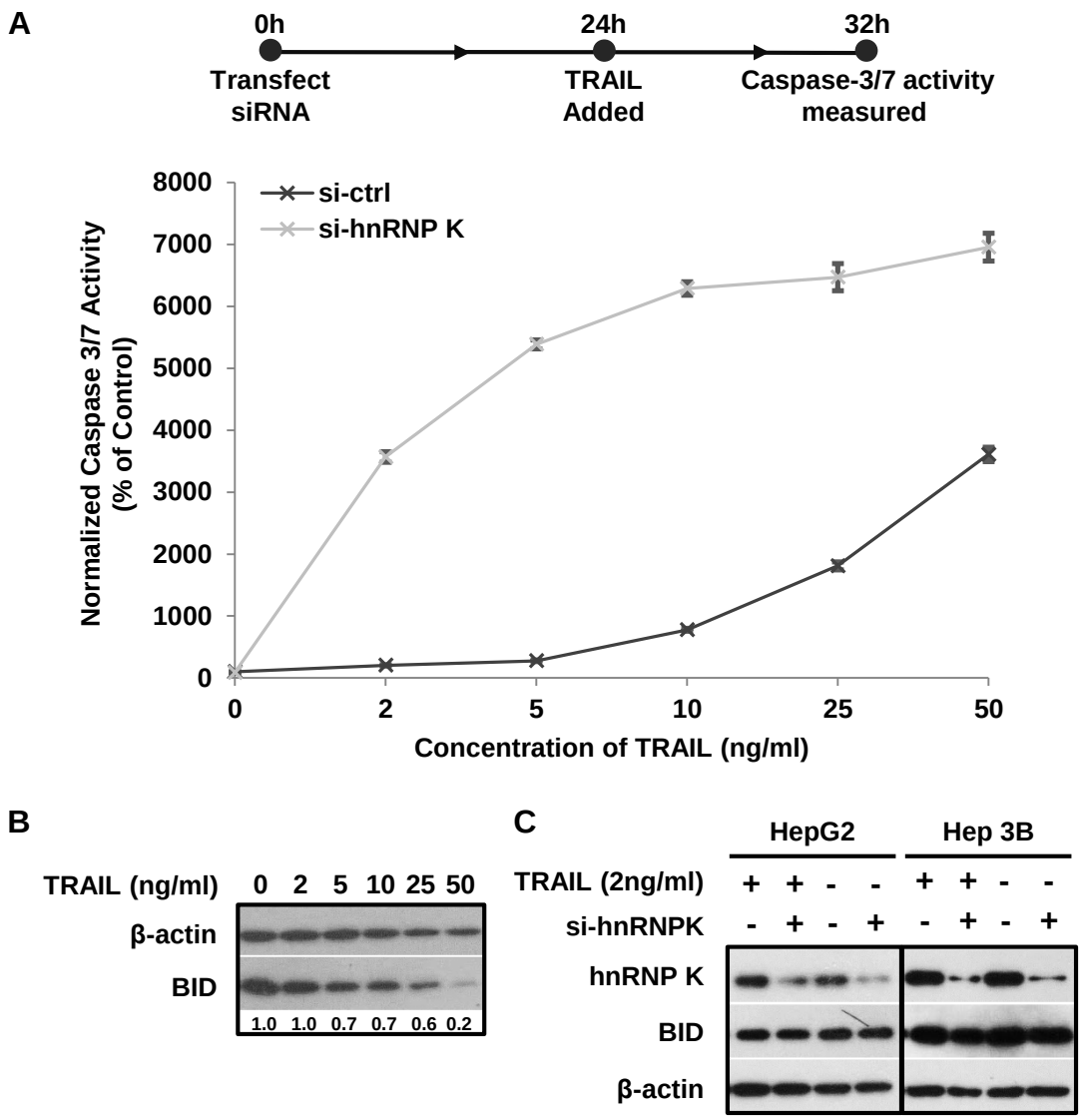


**Figure 4.7 Enhanced TRAIL-induced caspase-3/7 activity by hnRNP K downregulation is independent of BID cleavage**

**(A)** Enhancement in TRAIL-induced caspase-3/7 activity by hnRNP K downregulation was examined in HepG2, where 40 nM of si-ctrl or si-hnRNP K transfected cells for 24 h were treated with increasing concentrations of TRAIL for 8 h before caspase-3/7 activity was measured. Data expressed as normalised caspase-3/7 activity relative to cells transfected with si-ctrl and untreated with TRAIL and presented as mean ± SD% of triplicates. **(B)** The extent of BID cleavage in HepG2 treated with the indicated TRAIL concentrations was examined by western blot analyses of full-length BID protein level with β-actin as loading control. Intensities of full length BID protein level were measured with ImageJ, normalised to β-actin, expressed relative to that untreated with TRAIL and indicated beneath each band. **(C)** hnRNP K and full-length BID protein levels with β-actin as loading control were examined with western blot in HepG2 and Hep 3B that were transfected with 40 nM si-ctrl or si-hnRNP K for 24 h prior to 8 h incubation with 2 ng/ml of TRAIL.

As 2 ng/ml of TRAIL generated similar amount of caspase-3/7 activity in si-hnRNP K transfected cells as compared to si-ctrl transfected cells treated with 50 ng/ml TRAIL (Figure 4.7A), the amount of full length BID was examined in HepG2 with hnRNP K downregulated and treated with 2 ng/ml of TRAIL. Interestingly, it was observed in Figure 4.7C that the levels of full length BID were similar in differentially transfected HepG2 cells treated with 2 ng/ml of TRAIL regardless of hnRNP K downregulation. In addition, the level of full length BID in HepG2 with hnRNP K reduced and treated with 2 ng/ml of TRAIL (Figure 4.7C, left panel) was significantly higher than that in HepG2 cells treated with 50 ng/ml of TRAIL (Figure 4.7B). Likewise, when the level of BID in differentially transfected Hep 3B cells treated with 2 ng/ml of TRAIL for 8 h were analysed with western blot, the levels of full length BID were also similar in the differentially transfected cells (Figure 4.7C, right panel). These indicated that the observed enhancement in TRAIL-induced caspase-3/7 activity by hnRNP K downregulation was independent of BID cleavage and thus suggested it to be a direct effect from caspase-8 signalling to caspase-3/7.

Therefore, to investigate whether the enhanced TRAIL-induced caspase-3/7 activity was a direct effect of increased caspase-8 activity, HCC cells with hnRNP K downregulated and treated with low dose of TRAIL (as outlined in Figure 4.4A) had their caspase-8 activity measured.

The caspase-8 assay results demonstrated an increase in TRAIL-induced caspase-8 activity in both Hep 3B and HepG2 that had hnRNP K downregulated as compared to control. With a reduction in hnRNP K, the increase in caspase-8 activity following TRAIL treatment for 2 h and 8 h was 48.7% and 115.3% respectively in Hep 3B (Figure 4.8B) and 296.4% and 338.0% respectively in HepG2 cells (Figure 4.8C). The results were in agreement and correlated well with the enhanced TRAIL-induced

caspase-3/7 observed following hnRNP K reduction demonstrated earlier in this study (Figure 4.4B and C), strongly suggesting that the enhanced caspase-3/7 activity was a direct result from the increased caspase-8 activity.

Moreover, levels of full length caspase-8 in the differentially transfected cells were also examined with western blot and demonstrated to be expressed to a similar extent, indicating that hnRNP K downregulation did not affect the expression levels of caspase-8 (Figure 4.8A). This eliminates the possibility that the increase in TRAIL-induced caspase-3/7 activity was due to an increase in the expression level of caspase-8 upon hnRNP K downregulation. Taken together, the results indicate that the enhanced TRAIL-induced caspase-3/7 activity following hnRNP K downregulation is likely contributed by the direct increase in caspase-8 activity.

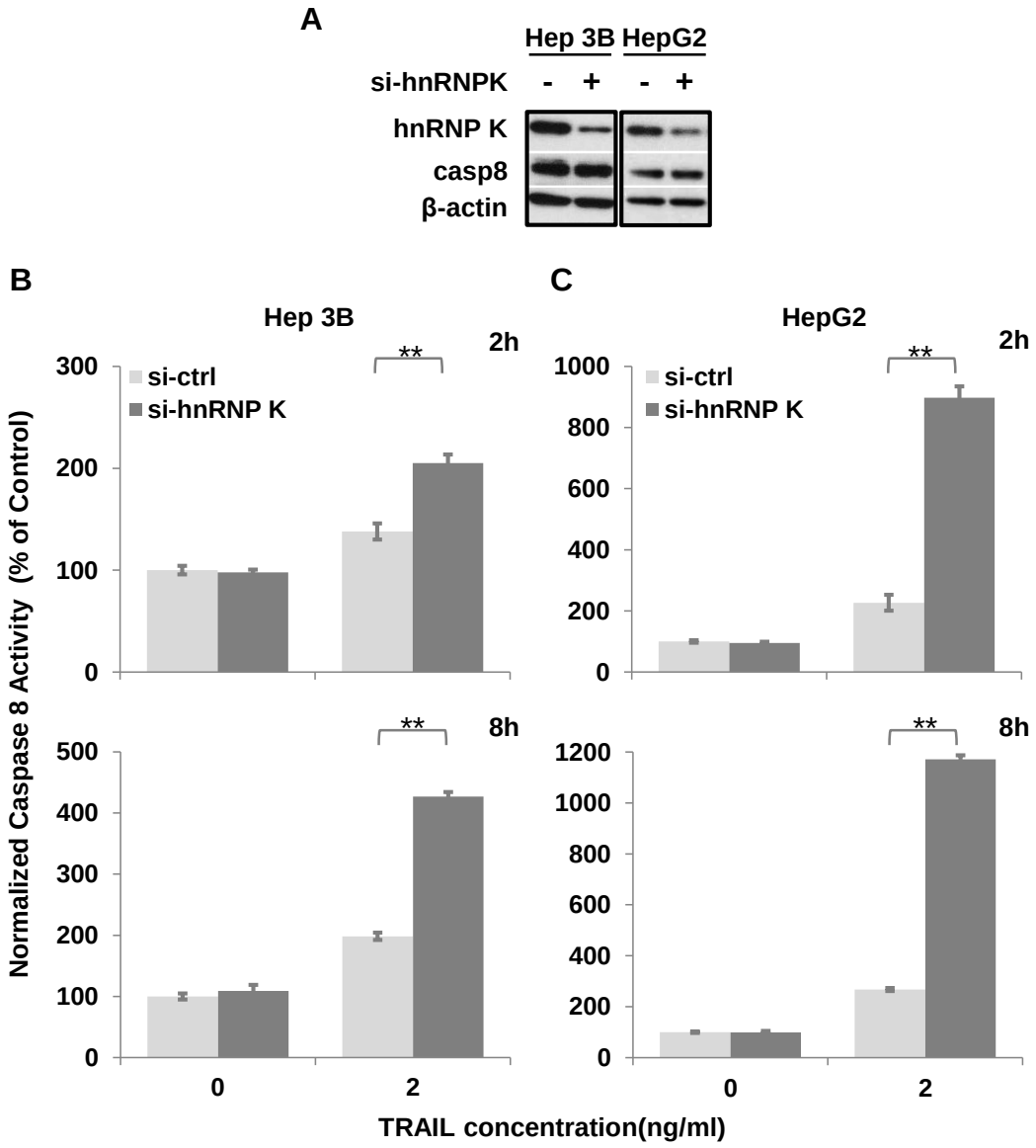


**Figure 4.8 Enhanced TRAIL-induced caspase-8 activity following hnRNP K downregulation**

(**A**) hnRNP K and full-length caspase-8 protein levels with β-actin as loading control in HepG2 and Hep 3B that were transfected with 40 nM si-ctrl or si-hnRNP K for 24 h were examined with western blot. Caspase-8 activity in (**B**) Hep 3B and (**C**) HepG2 cells with hnRNP K downregulated and treated with 2 ng/ml TRAIL for 2 h and 8 h was examined. Data was expressed as normalised caspase-8 activity relative to cells transfected with si-ctrl and untreated with TRAIL and presented as mean ± SD% of triplicates from a representative experiment of at least two with similar trend. (**$p$<0.01)

## 4.4 Reduction of hnRNP K downregulates the mRNA expression levels of anti-apoptotic genes

### 4.4.1 Identifying potential hnRNP K downstream targets associated with caspase-8 signalling pathway

Having shown that the enhanced TRAIL-induced caspase-3/7 activity caused by hnRNP K downregulation is independent of BID cleavage and likely contributed by the direct increase in caspase-8 activity, it was postulated that mediators involved in caspase-8 signalling or the extrinsic apoptotic pathway likely contributed to the increased TRAIL sensitivity resulting from hnRNP K downregulation. Hence, coupled with the knowledge that hnRNP K functions as a transcription factor, it was next examined whether the reduction of hnRNP K could have affected the expression levels of the mediators involved in the above-mentioned pathways. For this purpose, hnRNP K was first downregulated with siRNA in Hep 3B cells for 24 h and 48 h. Following which, RNA was extracted and gene expression profiles of the differentially transfected cells were examined with the use of exon array and analysed as detailed in Chapter 2.

To specifically uncover the genes involved in the apoptotic pathway associated with caspase-8 signalling that are likely to be regulated by hnRNP K, data from exon array analysis were analysed specifically for genes involved in the extrinsic apoptotic pathway and/or interact with caspase-8. As such, genes that were annotated to interact with caspase-8 as well as being involved in the extrinsic apoptotic pathway were first collated from BioGRID[3.1] as well as genes listed under gene ontology term 'Extrinsic Apoptotic Signaling Pathway' with accession GO:0097191. From the compilation, the expression profiles of a total of 109 genes could be identified from the exon array data. These 109 genes were then categorised into anti-apoptotic or pro-apoptotic function based on the classification by gene ontology terms 'Negative regulation of apoptotic

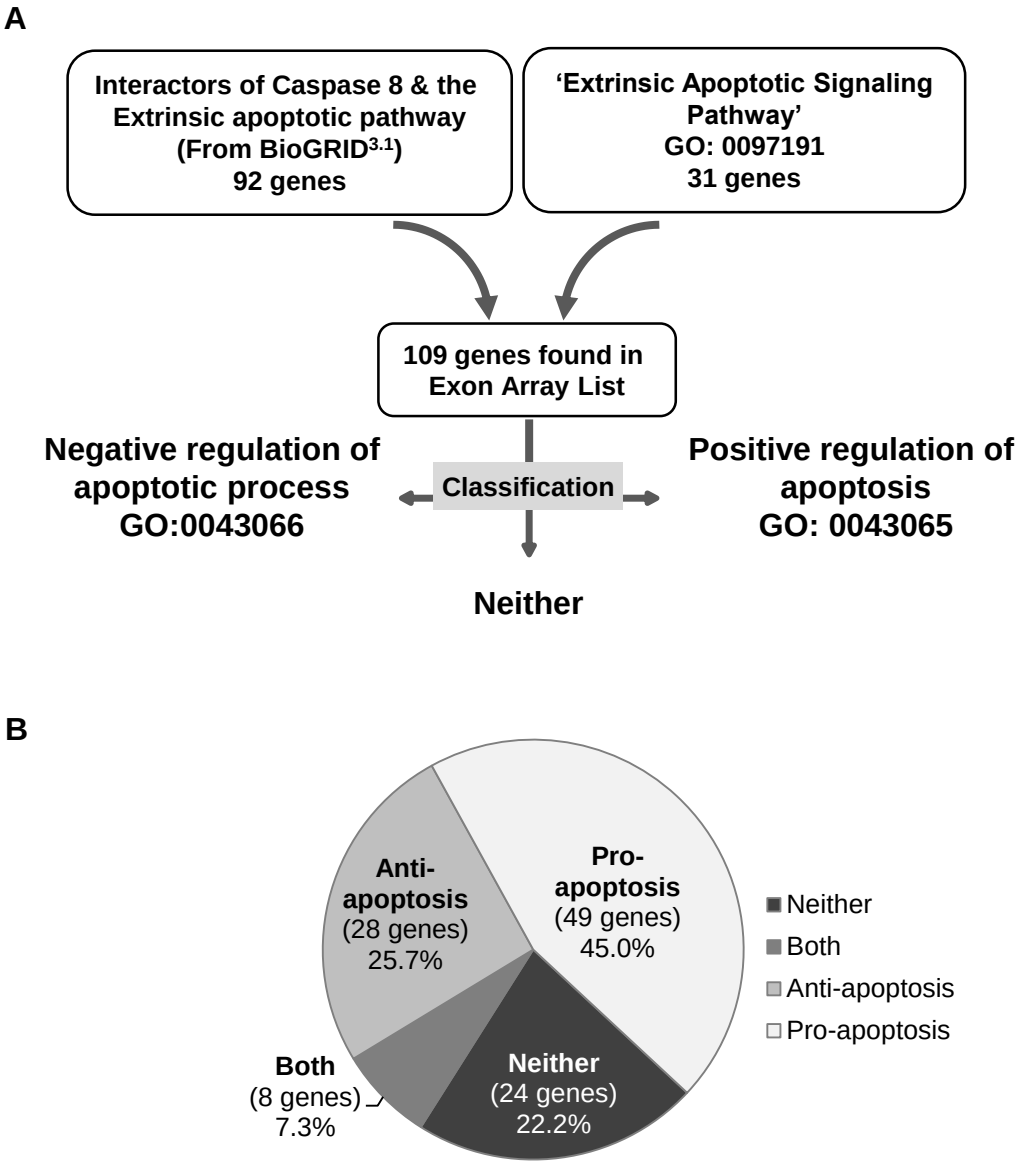


**Figure 4.9 Work process in elucidating genes in the extrinsic apoptotic pathway or interactors of caspase-8 that are regulated by hnRNP K**

**(A)** Flowchart depicting the steps in work process. Genes involved in the extrinsic apoptotic pathway as well as interactors of caspase-8 were collated and 109 genes with expression profiles present in exon array data yielded from Hep 3B cells transfected with 40 nM of si-ctrl or si-hnRNP K for 24 h and 48 h were further analysed. These genes were further classified into anti-apoptosis, pro-apoptosis, both or neither based on Gene Ontology's terms and ID as indicated as well as manual classification. **(B)** Pie-chart representing the number of selected genes in each classification as described above.

process' (GO: 0043066) and 'Positive regulation of apoptosis' (GO:0043065) coupled with subsequent manual classification (Figure 4.9A). Through this categorization, these genes were classified as pro-apoptosis, anti-apoptosis, neither (anti- nor pro-apoptosis) or both (anti- and pro-apoptotic) as shown in Figure 4.9B and listed in Table 4.1.

Upon attaining the list of anti- and pro-apoptosis genes, their expression profiles from the exon array data were examined. Gene expression values with a log2 fold change between -0.2 and 0.2 were classified as unchanged and the genes were subsequently ranked in order by their expression values at 24 h followed by that in 48 h of hnRNP K downregulation. In addition, only genes with consistent progressive change from 24 h to 48 h of hnRNP K reduction were selected for further analyses. Given that hnRNP K downregulation has been demonstrated earlier on in current study to induce apoptosis, genes of interest would thus be anti-apoptotic genes that were downregulated and pro-apoptotic genes that were upregulated following the downregulation of hnRNP K.

Having applied these criteria, genes in both pro- and anti-apoptosis categories that revealed progressive change from 24 h to 48 h post-transfection are depicted in Figure 4.10A and B respectively. Intriguingly, *TNFSF10* that encodes for TRAIL protein was the pro-apoptotic gene that had the highest gene expression as reflected by exon array data, at both 24 h and 48 h post hnRNP K downregulation (Figure 4.10A). As for the anti-apoptotic genes, *BIRC3*, *CFLAR* and *XIAP* had lowered gene expression at 24 h post-transfection. Of these three genes, *BIRC3* and *XIAP* are members of the IAP family (Srinivasula and Ashwell, 2008) and *CFLAR* has been demonstrated by a previous study to be a downstream target of hnRNP K (Chen et al., 2010).

Taking into consideration that the enhancement of TRAIL-induced caspase-3/7 activity was observed following 24 h of hnRNP K downregulation, pro-apoptotic gene *TNFSF10* that was observed to have the strongest gene expression, as well as *BIRC3*, *CFLAR* and *XIAP* that were observed to have decreased gene expression at 24 h post-hnRNP K reduction, were selected for subsequent downstream quantitative real-time PCR validation.

**Table 4.1 Classification of the 109 collated genes by their pro- or anti-apoptotic function**

| Function: | Pro-apoptosis | Anti-apoptosis | Neither* | Both# |
|---|---|---|---|---|
| | APAF1 | AIF1 | BCAP31 | BCL2L1 |
| | BAD | ARHGDIA | CASP1 | CAV1 |
| | BCL10 | BAG4 | CHD3 | FAS |
| | BID | BCL2 | CHUK | MCL1 |
| | CASP10 | BIRC2 | EZR | RIPK1 |
| | CASP14 | BIRC3 | FYN | RIPK2 |
| | CASP2 | CBL | HDAC7 | SQSTM1 |
| | CASP3 | CFLAR | JAK1 | UBC |
| | CASP4 | GDNF | KHDRBS1 | |
| | CASP6 | HIPK3 | MAP1LC3A | |
| | CASP7 | HTT | MAP1LC3B | |
| | CASP8 | IKBKB | MSN | |
| | CASP8AP2 | ITCH | NACA | |
| | CASP9 | KRT18 | NR1H4 | |
| | CRADD | MAP3K14 | PPP2R1B | |
| | CYLD | NOL3 | RALBP1 | |
| | DAP3 | PAK2 | RALGDS | |
| | DAXX | PARK7 | RET | |
| | DEDD | PEA15 | RYBP | |
| | DEDD2 | PRKCZ | SUMO1 | |
| | FADD | RHOA | TFCP2 | |
| | FAF1 | SIAH2 | USP2 | |
| | FASLG | SRC | VIM | |
| | HIP1 | TNFAIP3 | ZMYND11 | |
| | IFT57 | TRAF1 | | |
| | IKBKG | TRAF2 | | |
| | JAK2 | TSC22D3 | | |
| | LCK | XIAP | | |
| | LTBR | | | |
| | MAPK8 | | | |
| | MIB1 | | | |
| | MLLT11 | | | |
| | NLRC4 | | | |
| | NOD1 | | | |
| | PML | | | |
| | PTEN | | | |
| | RIPK3 | | | |
| | SH3RF1 | | | |
| | STAT1 | | | |
| | TNF | | | |
| | TNFRSF10A | | | |
| | TNFRSF10B | | | |
| | TNFRSF12A | | | |
| | TNFRSF1A | | | |
| | TNFRSF21 | | | |
| | TNFSF10 | | | |
| | TRADD | | | |
| | TRAF3 | | | |
| | UNC5B | | | |

* Neither pro- nor anti-apoptotic; # Both pro-and anti-apoptotic activities described

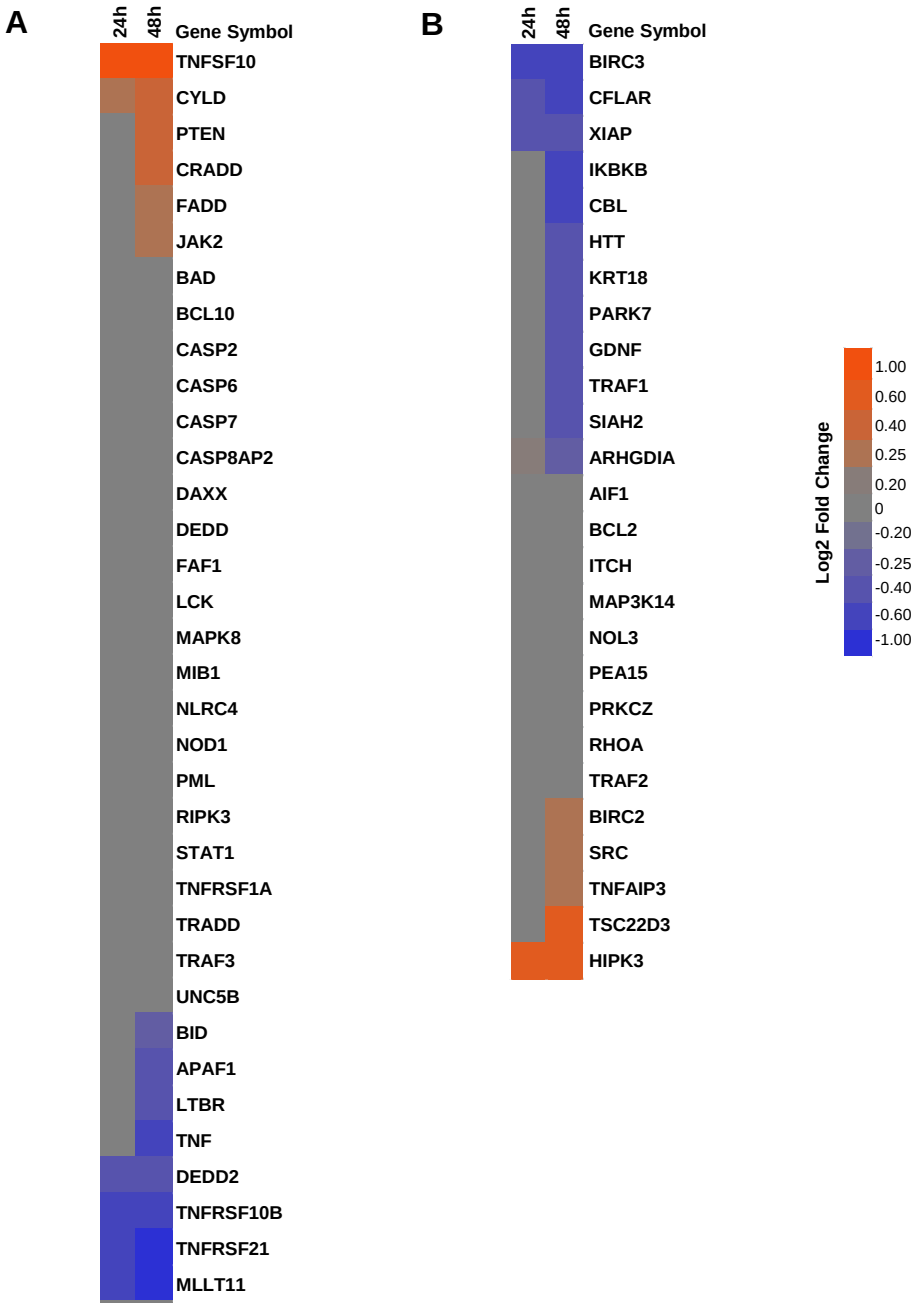


**Figure 4.10 Heatmap of selected pro- and anti-apoptotic genes that revealed progressive expression change following hnRNP K downregulation**

Expression profiles of selected genes that had been classified as **(A)** pro-apoptosis and **(B)** anti-apoptosis showing progressive expression change over the two time points (24 h and 48 h post-hnRNP K knockdown) from the exon array data. Orange and blue represent the increase and decrease in gene expression level and the intensity of colour correlates to the log2 fold change as indicated. Genes with no change in expression (grey) are also included.

### 4.4.2 Real-time quantitative PCR validation of potential hnRNP K regulated genes

Real-time quantitative PCR validation for the four selected genes was carried out. As the enhancement of TRAIL-induced caspase-3/7 activity caused by 24 h of hnRNP K downregulation was observed in both cell lines, these selected genes were validated in both Hep 3B and HepG2 cells with primers designed to amplify region that spans across exon-exon junction of each gene. In addition, for a gene to be potentially regulated by hnRNP K, the mRNA expression of the gene of interest should reflect similar expression trend in both HCC cell lines.

As revealed in Figure 4.11A, the mRNA fold expression level of the pro-apoptotic *TNFSF10* was upregulated in Hep 3B but downregulated in HepG2 cells following 24 h of hnRNP K downregulation. This upregulation of *TNFSF10* at the mRNA level in Hep 3B was consistent with the gene expression profile of *TNFSF10* in exon array (Figure 4.10A), but this increase in *TNFSF10* mRNA expression was lower than expected as its log2 fold change in the selected exon array data was the highest. Nonetheless, as its expression trend was disparate in both cell lines, *TNFSF10* was not selected for further analysis.

The mRNA expression levels of the anti-apoptotic genes were next investigated. Apart from *BIRC3* and *XIAP*, *BIRC2* that is also a member of the IAP family (Srinivasula and Ashwell, 2008) was similarly found in the list of anti-apoptotic genes seen in Figure 4.10B, but with no expression change at 24 h post-hnRNP K downregulation as revealed by the exon array data. Hence, the mRNA expression level of *BIRC2* was also investigated to determine whether its mRNA expression level correlated with the gene expression profile observed in exon array. The results revealed that the mRNA expression level of *BIRC2* was not significantly altered following hnRNP K downregulation for 24 h in both HCC cell lines (Figure 4.11B),

which was consistent with the observed gene expression profile from the exon array data analysis demonstrated in Figure 4.10B, and also indicated that *BIRC2* served as a good negative control.

From Figure 4.11C, it was observed that all three anti-apoptotic genes, *BIRC3*, *CFLAR* and *XIAP* were significantly downregulated at the mRNA level in both HCC cell lines following 24 h of hnRNP K downregulation, which was consistent with the results from the exon array (Figure 4.10B). This downregulation of *CFLAR* mRNA expression following 24 h of hnRNP K downregulation was expected as it has been previously demonstrated to be a direct downstream target of hnRNP K (Chen et al., 2010), and hence indirectly served as a positive control. On the contrary, it was demonstrated for the first time that the other two anti-apoptotic proteins of the IAP family, *BIRC3* and *XIAP*, were downregulated at the mRNA level following the reduction of hnRNP K.

Taken together, the results have indicated the reliability of the selected exon array data and suggested *BIRC3* and *XIAP* as potential downstream targets of hnRNP K that would require further study. With this in mind, XIAP that is the best characterised IAP thus far and deemed as the most potent caspase inhibitor (Srinivasula and Ashwell, 2008) was first chosen for further examination.

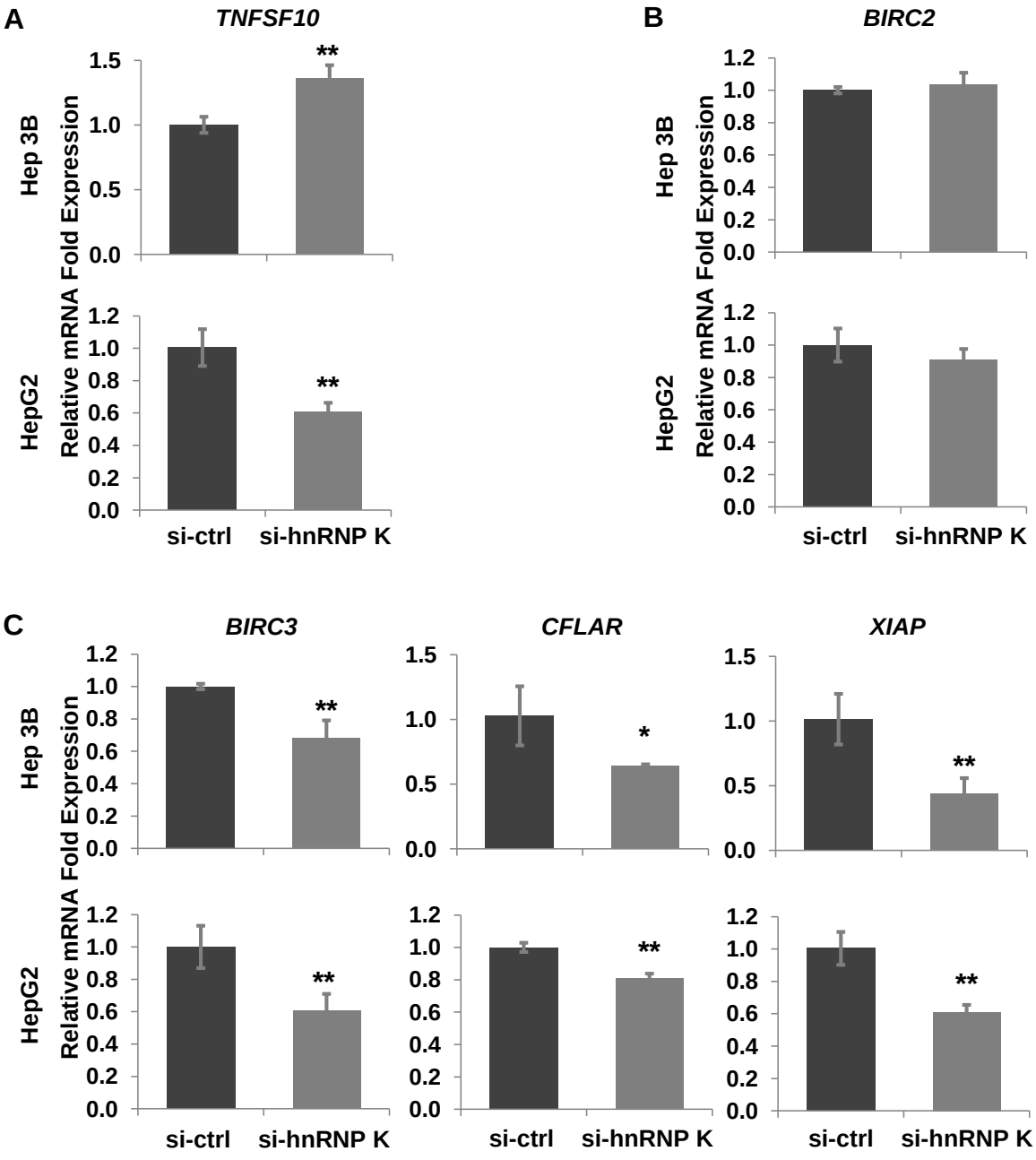


**Figure 4.11 Real-time quantitative PCR validation of selected genes based on exon array gene expression profile**

The mRNA expression levels of selected genes were examined in Hep 3B and HepG2 cells transfected with 40 nM si-ctrl or si-hnRNP K for 24 h with the use of real-time quantitative PCR. These genes were selected based on their function in apoptosis and gene expression data from exon array: **(A)** *TNFSF10* encoding for TRAIL, pro-apoptosis; **(B)** *BIRC2* serving as a negative control, anti-apoptosis; **(C)** Anti-apoptotic genes, *BIRC3* encoding for cIAP2, *XIAP* encoding for XIAP as well as the previously identified downstream target of hnRNP K, *CFLAR* that encodes for cFLIP. Data presented as mean ± SD of duplicates and expressed relative to control after normalisation to *HPRT* expression level. (**$p$<0.01, *$p$<0.05). Results are from a representative experiment of at least two with similar trend.

## 4.5 XIAP mRNA and protein levels are downregulated following hnRNP K reduction

X-linked IAP (XIAP) has been recognised as the most potent endogenous caspase inhibitor (Shiozaki and Shi, 2004) as it has been demonstrated to inhibit the activity of processed caspase-3/7 and sequester processed caspase-9 in a monomeric state (Shiozaki and Shi, 2004). Moreover, XIAP has been demonstrated to negatively affect caspase-8 and caspase-3 activities upon treatment with death ligands (Schile et al., 2008), indicating the pivotal role it possesses in inhibiting apoptosis.

Having shown that XIAP mRNA expression levels decreased following hnRNP K downregulation, it was next determined if there was any corresponding decrease in its protein levels. Western blot analyses in Figure 4.12 revealed that XIAP protein expression was similarly downregulated in both Hep 3B and HepG2 following 24 h as well as 48 h of hnRNP K downregulation. This indicated that the reduction of hnRNP K can result in a downregulation of XIAP protein, thereby decreasing the inhibition on caspase-3/7 as well as its activity. This hence likely contributed to the enhanced TRAIL-induced caspase-3/7 activity following hnRNP K reduction previously observed in Figure 4.4.

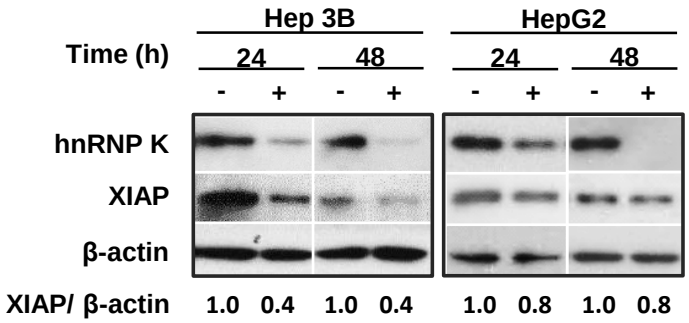


**Figure 4.12 hnRNP K downregulation reduces XIAP protein expression**
Reduction of XIAP protein levels following downregulation of hnRNP K in both HCC cell lines. β-actin served as a loading control in the total cell lysates of HepG2 and Hep 3B that were transfected with 40 nM si-ctrl (indicated by '-') or si-hnRNP K for 24 h and 48 h. Intensities of XIAP protein level, indicated beneath each band, were measured with ImageJ, normalised to β-actin's, expressed relative to cells transfected with si-ctrl at each time point.

Next, as a preliminary investigation to examine whether XIAP is a downstream transcriptional target of hnRNP K, the mRNA expression level of XIAP was quantitated with real-time PCR in Hep 3B cells with hnRNP K downregulated over time. The real-time quantitative PCR results in Figure 4.13A revealed that the mRNA level of XIAP was downregulated progressively over time following hnRNP K downregulation, indicating that the mRNA expression level of XIAP was dependent on the level of hnRNP K present.

However, due to the multifunctional property of hnRNP K, the observed downregulation in XIAP mRNA expression level could be a result of either decreased XIAP mRNA transcription or a decrease in XIAP mRNA stability following hnRNP K downregulation. Hence, to further elucidate whether XIAP is regulated by hnRNP K at the transcriptional level or at the post-transcriptional level, the stability of XIAP

mRNA following hnRNP K downregulation was examined. For this purpose, Hep 3B cells with hnRNP K downregulated for 48 h were treated with 5 μg/ml of Actinomycin D to block *de novo* mRNA synthesis and the level of XIAP mRNA was measured with quantitative real-time PCR at the various time points post-treatment as indicated. As observed in Figure 4.13B, the half-life of XIAP mRNA in control cells was similar to those cells with hnRNP K downregulated, with values of 5.9±0.3h and 6.2±0.1h respectively. This strongly suggests that the regulation of XIAP by hnRNP K did not occur at the post-transcriptional level but likely at the transcriptional level. This also indicated that the anti-apoptotic function of hnRNP K could in part be conferred by its ability to transcriptionally activate XIAP, which is a well-known anti-apoptotic protein.

Taken together, the results demonstrate that the anti-apoptotic function that hnRNP K possesses and exerts on the caspase-8 apoptotic signalling pathway is likely conferred by its ability to regulate various anti-apoptotic genes, *CFLAR, BIRC3 and XIAP*, that can inhibit the caspase-8 and caspase-3/7 axis in HCC cells.

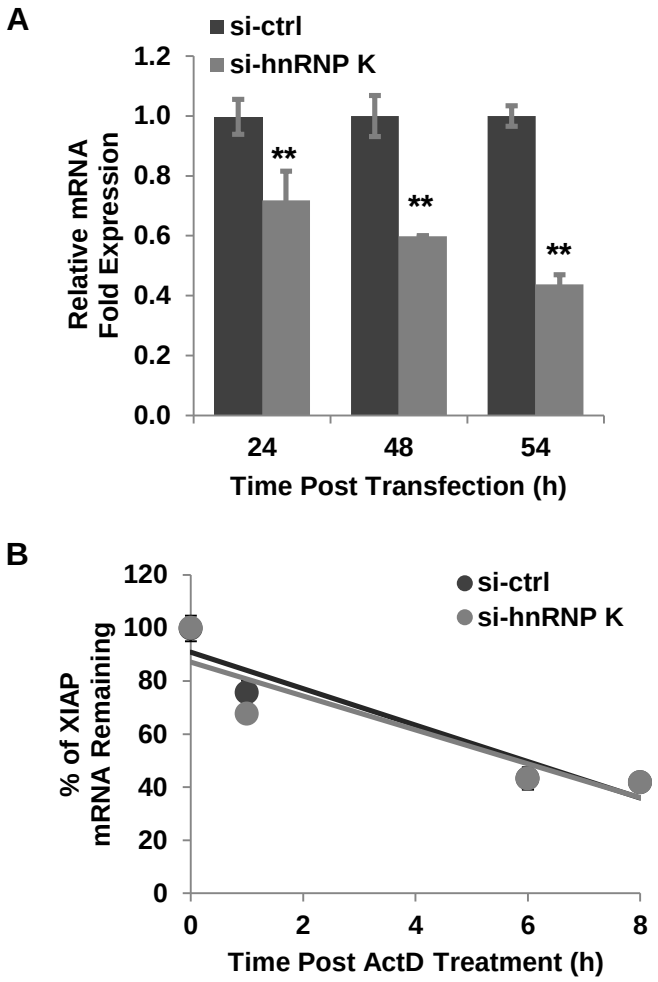


**Figure 4.13 XIAP as a potential downstream transcriptional target of hnRNP K**
**(A)** Real-time quantitative PCR analyses of XIAP mRNA level in Hep 3B cells transfected with 40 nM of si-ctrl or si-hnRNP K for the various time points indicated. Data presented as mean ± SD of duplicates and expressed relative to control after normalisation to *HPRT* expression level (**$p$<0.01). **(B)** The half-life of XIAP mRNA in Hep 3B with hnRNP K downregulated. The half-life of XIAP in Hep 3B cells transfected with 40 nM of si-ctrl or si-hnRNP K for 48 h were analysed with real-time quantitative PCR following treatment with Actinomycin D (Act D) for the time points indicated. Data presented as mean ± SD of duplicates and expressed relative to those without Act D treatment after normalisation to *HPRT* expression level. Results are from a representative experiment of at least two with similar trend.

## 4.6 Chapter Summary

Having shown that the induction of apoptosis occurred only after extended duration of hnRNP K downregulation in the previous chapter, the anti-apoptotic mechanism of hnRNP K was investigated in this chapter using HCC cell lines. By silencing caspase-8 or caspase-9 simultaneously with hnRNP K, caspase-8 was shown to be pivotal for the apoptosis observed at 72 h post-hnRNP K knockdown. As caspase-8 is a well-known initiator caspase of the extrinsic apoptotic pathway, the anti-apoptotic function of hnRNP K was examined with TRAIL that triggers the extrinsic apoptotic pathway. Intriguingly, a mere 24 h hnRNP K downregulation with siRNA enhanced TRAIL-induced caspase-3/7 activity dramatically, of which was hnRNP K-specific and did not involve any BID cleavage. Further analyses nonetheless revealed the increase in caspase-8 activity following hnRNP K downregulation, indicating that the anti-apoptotic effect of hnRNP K is likely exerted on the caspase-8 and caspase-3/7 axis (Figure 4.14).

To understand this novel hnRNP K-caspase-8 pathway, an exon array was performed on hnRNP K siRNA treated Hep 3B cells and the genes associated with caspase-8 signalling were selected for further analysis. Anti-apoptotic genes, *BIRC3*, *XIAP* and *CFLAR* were identified to have progressively reduced gene expression over time with the reduction of hnRNP K (Figure 4.14). Subsequent quantitative real-time PCR validation of these selected genes revealed their mRNA expression levels to be significantly downregulated following 24 h of hnRNP K downregulation, of which *BIRC3* and *XIAP* was demonstrated for the first time to be regulated by hnRNP K. XIAP in particular, a known potent inhibitor of caspase-3/7, was also selected for preliminary analysis in this study and was demonstrated to be downregulated at the protein level following hnRNP K downregulation. Moreover, results also

demonstrated that hnRNP K likely regulates XIAP at the transcriptional level. Together, these results strongly indicate that hnRNP K can exert its anti-apoptotic effect by regulating the expression levels of various anti-apoptotic genes at the caspase-8 and caspase-3/7 axis (Figure 4.14).

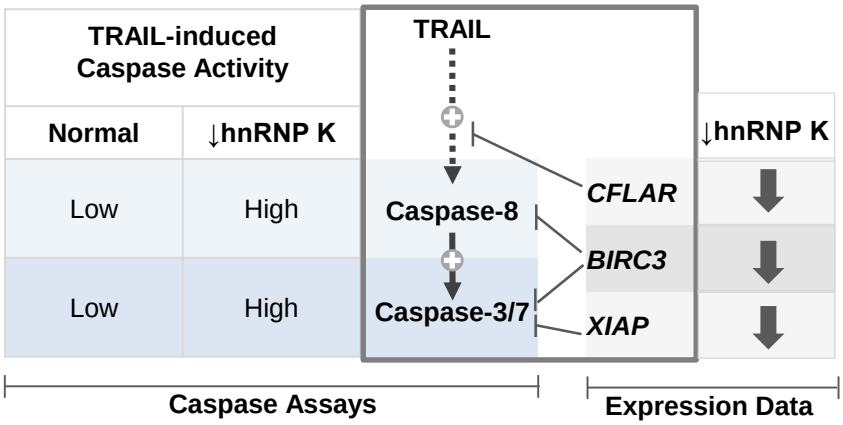


**Figure 4.14 hnRNP K regulates anti-apoptotic genes that inhibit the caspase-8 and caspase-3/7 axis**

Figure summarises results from Chapter 4. Results from the caspase assays have revealed enhanced TRAIL-induced caspase-8 and caspase-3/7 activities following hnRNP K reduction (↓hnRNP K), while data from the expression array and real-time PCR demonstrated the reduction of the anti-apoptotic genes that inhibit caspase-8 and caspase-3/7 activation. These indicated that hnRNP K can exert its anti-apoptotic effect by regulating the expression levels of various anti-apoptotic genes at the caspase-8 and caspase-3/7 axis. (⊕): activate; ( |— ) inhibit; (⬇) decrease expression.

# CHAPTER 5

## Discussion

## 5.1 hnRNP K inhibits apoptosis in HCC cells

hnRNP K is upregulated in various cancers including HCC, which is the third highest cause of cancer-related deaths globally (El-Serag and Rudolph, 2007) with apoptosis dysregulation as one of its hallmarks (Fabregat, 2009). Even though many studies have reported the elevated levels of hnRNP K in HCC (Table 3.1), its role in HCC has yet to be elucidated.

As hnRNP K has been demonstrated to transcriptionally regulate several oncogenes (Michelotti et al., 1995; Ritchie et al., 2003), its upregulation in various cancers has been deemed to contribute towards accelerated growth in cancer cells. Interestingly, recent studies have revealed an association between hnRNP K and apoptosis, albeit with contrasting results. Therefore, the role of hnRNP K in apoptosis induction was investigated with the use of HCC cell lines.

### 5.1.1 hnRNP K functions as an anti-apoptotic protein in HCC

A decrease in HCC cell viability was observed following hnRNP K downregulation (Figure 3.10 and 3.11), which is consistent with that previously reported (Inoue et al., 2007) and indicated the involvement of hnRNP K in cell survival. This decrease in cell viability is likely due to cell death, of which apoptosis might play a role, given earlier reports that suggested an association of hnRNP K expression levels with apoptosis. However, these reports were disparate, with Charroux et al. (1999) and Neo et al. (2005) reporting the association of hnRNP K overexpression with apoptosis, though more recent studies reported the downregulation of hnRNP K with apoptosis instead (van Domselaar et al., 2012; White et al., 2010).

Consequently, the involvement of hnRNP K downregulation with cell death was further investigated using *in vitro* functional analyses specific for different biochemical features of apoptosis (Table 3.3). The results established that the extended downregulation of hnRNP K, i.e. 72 h post-transfection, induced apoptosis in HCC cell lines (Figure 3.12A and 3.13). Interestingly, it was noted that increased hnRNP K downregulation was ineffective in bringing forward the initiation of apoptosis (Figure 3.13 and 3.14). Thus, it is hypothesised that hnRNP K functions as an anti-apoptotic protein where its downregulation at earlier time points, i.e. 24 h and 48 h post-transfection, may have compromised the cells' ability to inhibit apoptosis without the active initiation of apoptosis. Spontaneous induction of apoptosis was observed only after an extended duration of hnRNP K downregulation, but this trigger is still currently unclear.

The apoptosis induction observed following extended hnRNP K downregulation in HCC cell lines is in concordance with three recent studies that had similarly downregulated its expression levels in other cell lines; an increase in hypodiploid ($subG_0/G_1$) HT29 colorectal cancer cells was observed at 72 h post-transfection (Gao et al., 2009), increased caspase-3/7 activity and TUNEL positive cells in Sy5y neuroblastoma cells was reported at 72 h post-transfection (White et al., 2010), and an increase in annexin-V positive cells as well as caspase-3/7 activity was revealed in HeLa cells at 120 h post-transfection (van Domselaar et al., 2012). Taken together with the results from this study, it has been shown that apoptosis can be induced in various cell-types following hnRNP K downregulation, albeit with differences in duration of its reduction, which could be a cell-type specific response.

Given that extended hnRNP K downregulation led to apoptosis induction, the hypothesis that its reduction at earlier time points compromises cells' ability to inhibit

apoptosis concurred with a study which reported that hnRNP K downregulation in nasopharyngeal carcinoma (NPC) cell lines did not induce apoptosis, but sensitised the cells to death ligand-induced apoptosis (Chen et al., 2010). This also implies that the discrepancy in the required duration of hnRNP K downregulation for induction of apoptosis in different cells could be dependent on the availability and type of apoptotic stimuli present in each cell-type. Hence, these have together provided an explanation for the absence of cell viability changes or apoptosis induction observed in immortalised MRC5 lung fibroblast cell line despite extended hnRNP K downregulation (Moumen et al., 2005), as certain cell types may lack the apoptotic trigger or require an even longer period of hnRNP K reduction before apoptosis could be observed.

Therefore, it can be concluded that hnRNP K plays an important role in inhibiting apoptosis induction in various cell-types. Moreover, this study has demonstrated for the first time that hnRNP K likely serves as an anti-apoptotic protein that maintains the ability of HCC cells to inhibit apoptosis and ensure cell survival.

### 5.1.2 hnRNP K protein isoforms possess similar anti-apoptotic function

There are several hnRNP K protein isoforms (Dejgaard et al., 1994; Kimura et al., 2010), of which only isoform–a and –b are fully described. In an attempt to study hnRNP K's specificity in the apoptosis induced upon its downregulation with siRNA, isoform–a and –b expression plasmids were generated to restore its reduced levels (Figure 3.15 and 3.16). A decrease in extent of apoptosis was observed upon restoration of hnRNP K expression levels, thus demonstrating that the apoptosis induced following its reduction was indeed hnRNP K-specific and also further validated hnRNP K's role as an anti-apoptotic protein. However, restoration of hnRNP K levels was unable to completely abrogate the apoptosis induced (Figure 3.18 and 3.19). A plausible explanation would be that the siRNA treatment resulted in the downregulation of all hnRNP K isoforms, thus the use of either isoform–a or –b expression plasmids solely would be insufficient for total abrogation of the apoptosis induced. In line with the results shown in Figure 3.18 and 3.19, the stable overexpression of hnRNP K (isoform–a) in Sy5y cell line has been shown to suppress hnRNP K-downregulation-induced apoptosis, though the induction of apoptosis could not be inhibited completely (White et al., 2010).

It is noted that the restoration of hnRNP K expression levels with isoform–a resulted in a better inhibition of induced apoptosis when compared to isoform–b (Figure 3.18 and 3.19). However, it cannot be concluded that isoform–a exerted a stronger anti-apoptotic effect as compared to isoform–b as structural modelling of both hnRNP K isoforms' KH3 domain (inclusive of the C-terminals) has revealed no structural differences (Leopoldino et al., 2007). In terms of their function, it was reported in a study on HBV replication that there was no significant difference between either isoform in enhancing viral load (Ng et al., 2005). Thus, the observed

difference in suppression of hnRNP K-downregulation-induced apoptosis could possibly be due to a slightly higher expression level of isoform–a as compared to –b (Figure 3.17E and F).

In summary, this study has provided experimental evidence that both hnRNP K isoform–a and –b possess similar anti-apoptotic functions, maintaining HCC cells' ability to inhibit apoptosis. In addition, it has been demonstrated that the apoptosis induced as a result of hnRNP K reduction is indeed hnRNP K-specific.

## 5.2 The mechanism behind hnRNP K-downregulation-induced apoptosis in HCC

Although several studies have reported the induction of apoptosis following hnRNP K downregulation, the underlying mechanism has not been well elucidated. Based on the experimental findings presented in the previous section, it is hypothesised that hnRNP K is an anti-apoptotic protein where its downregulation at early time points (i.e. 24 h and 48 h) merely compromised the cell's ability to inhibit apoptosis, while the trigger behind the apoptosis induced following extended hnRNP K downregulation remains to be characterised. Thus, this has prompted a closer examination of hnRNP K's anti-apoptotic ability, as well as further investigation on the trigger for the induction of apoptosis.

### 5.2.1 Caspase-8 signalling is pivotal in triggering apoptosis in HCC cells following hnRNP K downregulation

When caspase-8 or -9 apoptotic signalling was assessed for their contribution in apoptosis induction following hnRNP K downregulation, the results intriguingly revealed the importance of caspase-8 in apoptosis initiation. The co-knockdown of hnRNP K and caspase-8 almost completely abrogated the activity of caspase-3/7 activity, while co-knockdown of hnRNP K and caspase-9 yielded an increase in caspase-3/7 activity instead (Figure 4.1). The mechanism behind the increase in caspase-3/7 activity following downregulation of both caspase-9 and hnRNP K is currently unknown, though heightened apoptosis following caspase-9 downregulation has been previously reported (Shah et al., 2004).

Nonetheless, it is revealed for the first time that the trigger initiating apoptosis functions via caspase-8 in HCC cells, hence demonstrating the importance of caspase-8 apoptotic signalling in hnRNP K-downregulation-induced apoptosis. This is in line

with the observation by a previous study that hnRNP K regulates the expression of cFLIP, a caspase-8 inhibitor (Chen et al., 2010). In contrast, White et al. (2010) revealed the translocation of PKCδ to the mitochondria following hnRNP K reduction, thus attributing the induction of apoptosis to the intrinsic apoptotic pathway. However, the activation of intrinsic apoptotic pathway would have allowed for caspase-3/7 to remain activated even in the absence of caspase-8, which is contrary to this study's findings. As discussed previously, such discrepancy could be a cell-type specific effect. Hence, while it has been demonstrated that caspase-8 apoptotic signalling is important for the initiation of apoptosis following hnRNP K reduction in HCC cells, other possible sources of apoptosis initiators such as reactive oxygen species production following extended durations of hnRNP K downregulation or cell-type specific differences should not be disregarded (van Domselaar et al., 2012).

Although current findings have revealed the pivotal role of caspase-8 in apoptosis induction following hnRNP K reduction in HCC cells, the exact initiator that triggers the activation of caspase-8 remains to be elucidated. Nonetheless, as caspase-8 is directly involved in the extrinsic apoptotic pathway (Elmore, 2007), mediators in this pathway are likely candidates for the apoptosis trigger.

### 5.2.2 hnRNP K confers HCC cells with resistance to apoptotic stimuli

In line with the hypothesis that hnRNP K downregulation compromises the ability of HCC cells to inhibit apoptosis at earlier time points, it was revealed that the downregulation of hnRNP K was able to sensitise HCC cells to TRAIL-induced apoptosis as exemplified by the significant increase in caspase-3/7 activity (Figure 4.4). This is consistent with previous findings in which an increase in TUNEL positive cells was reported following 48 h of hnRNP K downregulation and concurrent TRAIL treatment in NPC cells (Chen et al., 2010). Such sensitization to cell death following hnRNP K downregulation is not limited to death-ligand induced apoptosis only, as a recent study has also demonstrated that the loss of hnRNP K could sensitise HeLa cells to cytotoxic lymphocyte-mediated killing (van Domselaar et al., 2012).

The anti-apoptotic effect of hnRNP K in HCC cells was also further demonstrated, where increasing concentrations of hnRNP K siRNA were able to specifically increase TRAIL-induced caspase-3/7 activity in a dose-dependent manner (Figure 4.5). Conversely, the overexpression of hnRNP K was able to reduce TRAIL-induced caspase-3/7 activity significantly (Figure 4.6). The overexpression of either hnRNP K isoforms resulted in a similar reduction in TRAIL-induced caspase-3/7 activity, further supporting the deduction that both isoforms possess similar anti-apoptotic capabilities. Thus, these findings have indicated that hnRNP K confers HCC cells with the ability to resist apoptosis. In addition, as HCC cells have been reported to be resistant to apoptosis mediated by several death receptors (Fabregat, 2009), results from this study have also suggested the possibility of sensitizing HCC cells to death ligand-induced apoptosis via hnRNP K downregulation.

### 5.2.3 hnRNP K regulates expression of anti-apoptotic genes

hnRNP K is a multifunctional protein involved in various cellular processes and its nuclear functions include that of a transcriptional factor and a regulator for alternative splicing (Bomsztyk et al., 2004). In recent years, several studies have implicated hnRNP K in the alternative splicing of apoptotic proteins (Revil et al., 2009; Venables et al., 2008). Notably, anti-apoptotic protein cFLIP has been demonstrated to be a direct downstream transcriptional target of hnRNP K (Chen et al., 2010), indicating that hnRNP K could directly regulate the expression of genes involved in apoptosis as a transcription factor.

In this study, the correlation between hnRNP K's role as a transcription factor and its observed anti-apoptotic function was examined. The experimental results revealed a significant decrease in hnRNP K expression in the nucleus upon its reduction with siRNA (Figure 3.10), suggesting that hnRNP K's nuclear functions are likely to be affected following siRNA treatment. Coupled with the importance of caspase-8 apoptotic signalling in HCC cells elucidated in this study, the expression of genes involved in the extrinsic apoptotic pathway as well as interactors of caspase-8 were examined based on an exon array performed with the use of siRNA-treated Hep 3B (Figure 4.9). In view of the fact that hnRNP K affects the expression of other transcription factors such as c-Myc (Michelotti et al., 1996) and the androgen receptor (Wang et al., 2008), emphasis was placed on genes that showed greatest differential expression at 24 h post-hnRNP K downregulation to exclude possible unwanted secondary effects of hnRNP K downregulation on gene expression.

Intriguingly, the results revealed that the three anti-apoptotic genes with the greatest expression reduction in the exon array, *BIRC3* (cIAP2), *CFLAR* (cFLIP) and *XIAP* (XIAP), were all downregulated at the mRNA level following hnRNP K

downregulation in both HCC cell lines tested (Figure 4.10 and 4.11). This indicated that hnRNP K positively regulates the expression level of these three anti-apoptotic genes, of which the observations for cFLIP were consistent with a previous study (Chen et al., 2010). Nonetheless, this is the first demonstration of cIAP2 and XIAP gene expression regulation by hnRNP K.

In addition, preliminary analyses examining the regulation of XIAP by hnRNP K have revealed a decrease in its protein levels following hnRNP K downregulation (Figure 4.12), and also demonstrated that hnRNP K likely affects XIAP's expression at the transcriptional level (Figure 4.13). These findings strongly indicated that hnRNP K transcriptionally regulates XIAP but future validation with in-depth promoter analyses for XIAP would be required to affirm it as a direct downstream target of hnRNP K. However, hnRNP K can transcriptionally regulate genes via binding to a CT-rich region on promoters such as that for c-Src, c-Myc, thymidine kinase, neuronal acetylcholine receptor β4 subunit (Michelotti et al., 1996; Ritchie et al., 2003; Lynch et al., 2005; Hsieh et al., 1998; Du et al., 1998) or in the absence of direct DNA contact such as that observed for COX-2, p21 and MDM2 (Shanmugam et al., 2008; Moumen et al., 2005) or through interfering the activity of other transcription factors (Denisenko et al., 1996; Miau et al., 1998). As such, it is only with the identification of the exact promoter region on XIAP that hnRNP K exerts its positive regulative effect, before it could be systematically uncovered whether hnRNP K transcriptionally regulates XIAP via direct contact to a CT-rich region or through other transcription factors. This could also be further substantiated with additional validation studies, such as 'rescue experiments' by overexpressing XIAP following hnRNP K depletion, where the overexpression of XIAP that is a potent inhibitor of effector caspases is most likely to extensively reduce the extent of induced apoptosis.

Similarly, these future validation studies can also be applied to cIAP2 identified from this study. Nonetheless taken together, the experimental results have demonstrated that hnRNP K is an upstream regulator of the three anti-apoptotic genes that can contribute to the maintenance of HCC cells' ability to inhibit apoptosis.

Having demonstrated that the three anti-apoptotic genes involved in the extrinsic apoptotic pathway are regulated by hnRNP K, this has shed light on the p53-independent apoptosis observed following hnRNP K downregulation (Figure 3.12 and 3.14). As the extrinsic apoptotic pathway is the most well-established p53-independent apoptotic pathway (Ashkenazi, 2008), the demonstration that hnRNP K regulates the above-mentioned anti-apoptotic proteins has strongly suggested that the p53-independent apoptosis observed following hnRNP K downregulation is via the extrinsic apoptotic pathway. This further supports the proposal that apoptosis observed following hnRNP K reduction was initiated by a trigger that signals via the extrinsic apoptotic pathway.

Therefore, experimental findings from this current study have collectively revealed the underlying mechanism of apoptosis induction observed in HCC cells. As shown in Figure 5.1, though hnRNP K downregulation at early time points of 24 h and 48 h did not induce apoptosis spontaneously, it reduced the cells' anti-apoptotic capability and allowed for the trigger of apoptosis that signals via the caspase-8 apoptotic pathway.

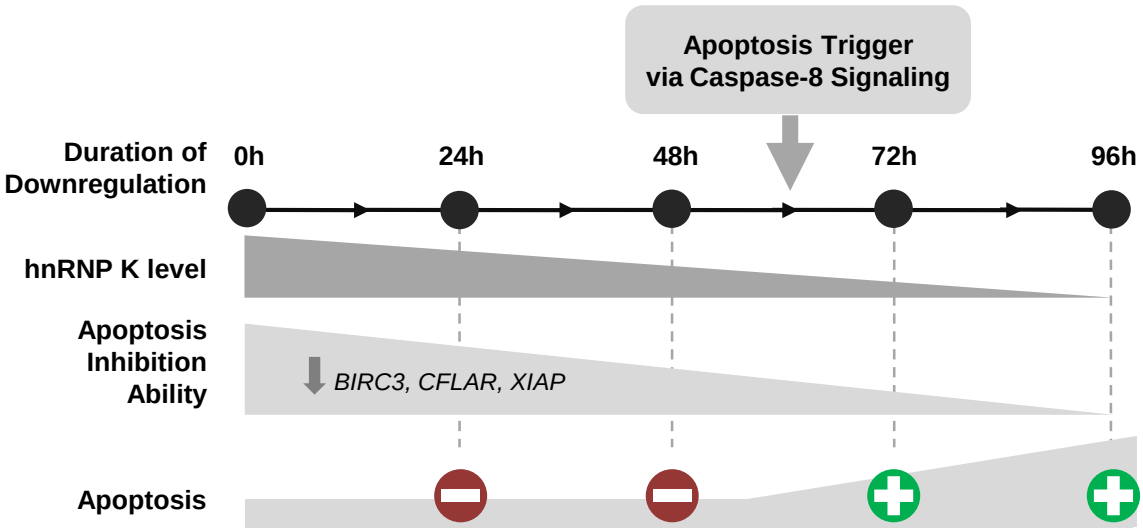


**Figure 5.1 Underlying mechanism behind hnRNP K-downregulation-induced apoptosis in HCC**

HCC cell survival requires the active inhibition of apoptosis. Downregulation of hnRNP K results in a decrease of HCC cells' ability to inhibit apoptosis, due to the reduction in anti-apoptotic proteins. Subsequent apoptotic trigger that signals via caspase-8 between 48 h and 72 h of hnRNP K downregulation results in apoptosis observed at 72 h post-transfection.

## 5.3 Dysregulated apoptosis in HCC as a result of elevated hnRNP K levels

Dysregulation of apoptosis is a part of the multistep event leading to the formation of aggressive HCC (Fabregat, 2009; Feitelson et al., 2002; Hussain et al., 2007). In HCC, the dysregulation of apoptosis is complex, involving a plethora of signalling pathways such as that of p53, survival signalling as well as various apoptotic pathways. In addition, HCC cells have been reported to be resistant to apoptosis mediated by several death receptor ligands at the extrinsic apoptotic pathway (Fabregat, 2009). Given that apoptosis dysregulation has been reported to be mainly due to upregulation of anti-apoptotic pathways, therapeutic strategies that selectively inhibit anti-apoptotic signals may aid in HCC treatment (Fabregat, 2009).

Hence, as hnRNP K has been demonstrated in this study to be an upstream regulator of the anti-apoptotic proteins cIAP2, cFLIP and XIAP, these proteins could likely contribute to the apoptosis resistance of HCC cells and thus hnRNP K's maintenance of the cells' anti-apoptotic ability.

cIAP2 is a multifunctional protein that is involved in many biological processes including apoptosis and regulation of NF-κB signalling. It possesses E3 ubiquitin-protein ligase activity, capable of ubiquitinating various substrates such as caspase-8 and RIPK1 (Gyrd-Hansen and Meier, 2010; Tenev et al., 2011). In addition, cIAP2 has been demonstrated to be upregulated at the mRNA level in HCC cell line with integrated HBV genome (Lu et al., 2005) and upregulated at both mRNA and protein levels in recurrent HCC associated with HBV infection (Weng et al., 2012). Despite the association of cIAP2 with HCC in these two studies, there is currently no experimental evidence regarding its direct effect on HCC's resistance to apoptosis. Nevertheless, given cIAP2's anti-apoptotic functions (Gyrd-Hansen and Meier, 2010), it can be hypothesised that cIAP2 has a role in HCC's resistance to apoptosis by

contributing to the cells' anti-apoptotic ability maintained by hnRNP K. However, further studies would be required to evaluate its contribution in greater detail.

In contrast to cIAP2, there are more reports on cFLIP and XIAP playing an important role in the dysregulation of apoptosis in HCC. cFLIP is a major inhibitor of caspase-8 activation mediated by death receptors (Irmler et al., 1997) and has been reported to be upregulated at the mRNA level (Lee et al., 2008) as well as at the protein level in HCC samples and cell lines (Okano et al., 2003). The downregulation of cFLIP with siRNA was able to sensitise HCC cells to death receptor-mediated apoptosis (Ganten et al., 2004; Okano et al., 2003). Thus, this indicated that cFLIP likely confers HCC cells with resistance to apoptosis. It also lends support to this study's experimental findings that highlighted the importance of caspase-8 apoptotic signalling in hnRNP K-downregulation-induced apoptosis.

XIAP is a potent inhibitor of caspase-3, -7 and -9 (Holcik and Korneluk, 2001) and possesses E3 ubiquitin-protein ligase activity, capable of ubiquitinating various substrates such as caspase-8 and RIPK1 (Schile et al., 2008; Van Themsche et al., 2009). Apart from inhibiting apoptosis directly via caspase inhibition, XIAP can also cause the activation of JNK pathway as well as the subsequent activation of NF-κB, thereby promoting the survival pathways (Schimmer et al., 2006). Elevated XIAP mRNA levels have been reported in HCC samples and cell lines (Notarbartolo et al., 2004; Tao et al., 2004). In addition, immunostaining of XIAP has revealed its overexpression in HCC, where 70% of the HCC samples showed moderate to strong cytoplasmic staining for XIAP while the surrounding non-tumour tissues as well as normal liver tissues revealed a lack of or weak XIAP expression (Shiraki et al., 2003; Vischioni et al., 2006). Furthermore, XIAP has been revealed to be upregulated in nearly 90% of samples from advanced HCC patients, which correlated with apoptosis

resistance and enhanced in-vitro invasiveness in HCC cell lines as reported by Shi et al. (2008b). Similar to cFLIP, downregulation of XIAP has been reported to sensitise HCC cells to TRAIL-induced cell death (Pan et al., 2008; Yamaguchi et al., 2005). Hence, both cFLIP and XIAP are able to confer HCC cells with resistance to apoptosis, thus playing a crucial role in HCC's anti-apoptotic capability maintained by hnRNP K.

Based on the experimental evidence from various studies discussed above, coupled with this study's findings that hnRNP K reduction can result in the corresponding downregulation of both caspase inhibitors i.e. cFLIP and XIAP, it could be hypothesised that the enhancement in TRAIL-induced caspase-3/7 activity following hnRNP K downregulation (Figure 4.4) is a direct result of increase in caspase-8 activity. Indeed, it was demonstrated that TRAIL-induced caspase-8 activity increased (Figure 4.8), indicating that the reduction of cFLIP upon hnRNP K downregulation has resulted in the direct removal of inhibition at caspase-8. It is thus conceivable that the corresponding reduction of XIAP also brought about an increase in caspase-3/7 activity due to increased caspase-8 activity, hence bypassing the need for cleavage of BID to enhance caspase-3/7 activity. This is also supported by Jost et al. (2009), where it was demonstrated that the loss of XIAP resulted in Fas-induced apoptosis independent of BID in mouse hepatocytes. The reduction of inhibition at the caspase-8 and caspase-3/7 axis following hnRNP K downregulation is in line with the findings that hnRNP K influences the expression of cFLIP and XIAP and hence affects caspase activity indirectly.

Taken together, it is conceivable that a reduction in hnRNP K expression results in the downregulation of cFLIP and XIAP, which subsequently compromises the anti-

apoptotic machinery. This would allow for the sensitization of HCC cells to death ligands such as TRAIL via the caspase-8 and caspase-3/7 axis.

Therefore, with the results demonstrated in this study, a model of apoptosis resistance in HCC conferred by hnRNP K could be constructed as seen in Figure 5.2. hnRNP K plays a crucial anti-apoptotic role in HCC cells' resistance to apoptosis by maintaining high levels of caspase inhibitors cFLIP and XIAP, which can directly inhibit the activities of caspase-8 and caspase-3/7. In addition, the high levels of cFLIP serves as a blockade for the activation of extrinsic pathway by death ligands such as TRAIL that is constitutively produced in HCC and HCC cell lines (Shiraki et al., 2005). Upon the downregulation of hnRNP K, the levels of cFLIP and XIAP decreased and resulted in the removal of caspase inhibition, causing downstream apoptosis.

While many studies have reported the upregulation of hnRNP K in HCC (Table 3.1), it was only recently that an in-depth study was conducted on the expression level of hnRNP K in cirrhotic liver as well as early- and late-stage HCC samples, where the authors proposed the use of hnRNP K as a specific tissue biomarker for distinguishing early-stage HCC from liver cirrhosis and that a positive hnRNP K staining could be taken as an indicator of HCC (Guo et al., 2012). Liver fibrosis and cirrhosis arising as a consequence of chronic liver injury with constant occurrence of apoptosis and inflammation can lead to the subsequent formation of HCC, where damaged hepatocytes accumulate multiple genetic changes that increase their proliferation and allow for evasion of apoptosis (Chakraborty et al., 2012). These findings correlate well in indicating that the expression level of hnRNP K is low in cirrhotic liver where apoptosis is constantly occurring but increases in HCC that has developed the ability to evade apoptosis. Thus, as it was revealed in this study that hnRNP K possesses a

strong anti-apoptotic function at the caspase-8 and -3/7 axis in HCC, this indicated that hnRNP K could potentially serve as a prognostic marker to indicate the extent of apoptosis resistance in HCC, in addition to its role as a diagnostic marker proposed by Guo et al. (2012). This would allow for early diagnosis of early-stage HCC and could serve as a guide for the choice of treatment.

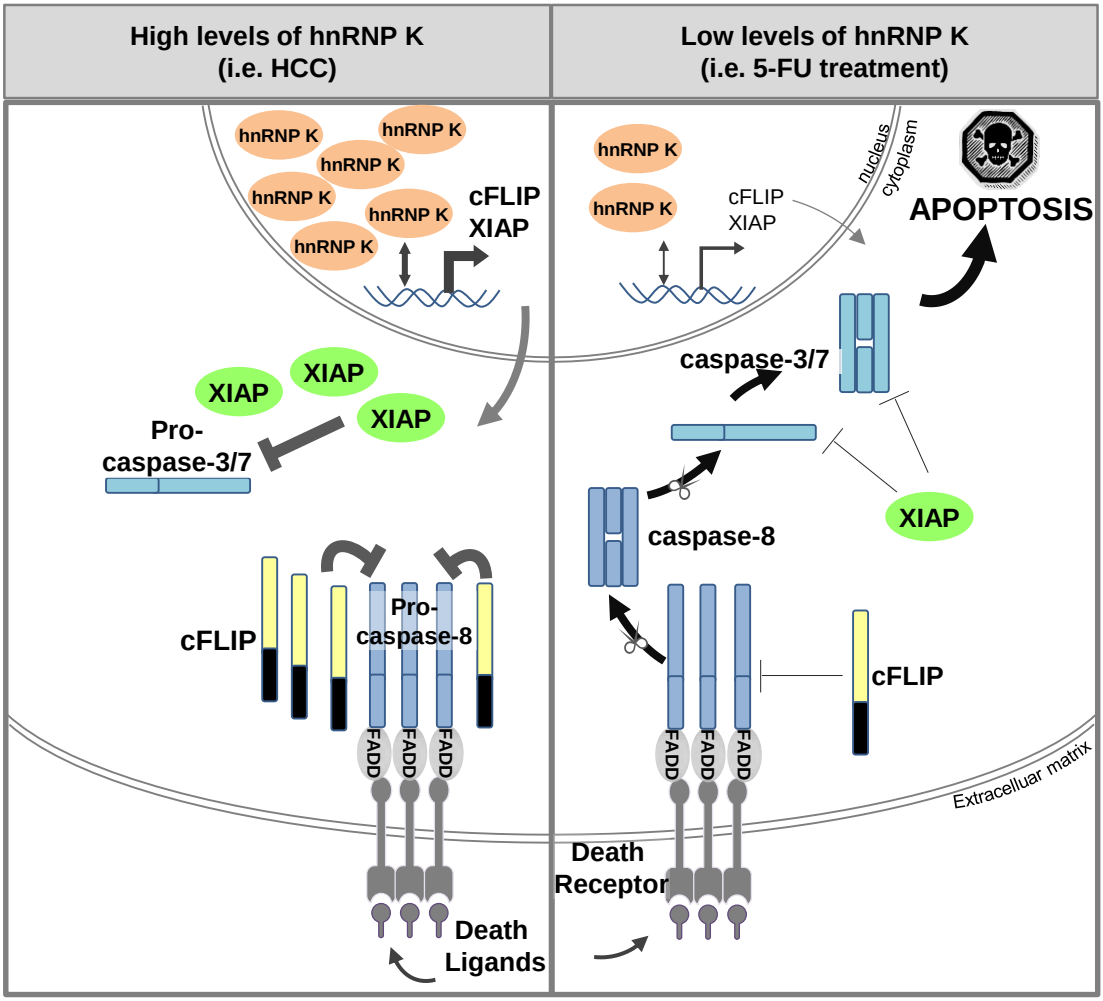


**Figure 5.2 A model of apoptosis resistance in HCC conferred by hnRNP K**

hnRNP K maintains apoptosis resistance of HCC by maintaining high levels of caspase inhibitors e.g. cFLIP and XIAP, which can directly inhibit the activities of caspase-8 and caspase-3/7. High levels of cFLIP and XIAP serve as blockade at the caspase-8-3/7 axis for the activation of extrinsic pathway by death ligands such as TRAIL that is constitutively produced in HCC and HCC cell lines. With hnRNP K reduction i.e. during chemotherapeutic drug treatment, the levels of cFLIP and XIAP decrease and result in the removal of caspase inhibition, thereby contributing to the apoptosis induction.

## 5.4 The physiological relevance of hnRNP K downregulation during chemotherapeutic drug treatment

Although elevated levels of hnRNP K have been constantly reported in various cancers as well as HCC (Table 3.1), various chemotherapeutic drugs that reduces cell viability have been shown to reduce the expression of hnRNP K in cancer cells, hence suggesting the association between reduced hnRNP K expression and cell death. The implications of this observed reduction in hnRNP K expression by chemotherapeutic drugs are discussed below.

### 5.4.1 hnRNP K downregulation as a contributor to 5-Fluorouracil-induced apoptosis

5-Fluorouracil (5-FU) is an anti-metabolite chemotherapeutic drug commonly used to treat various cancers, where it exerts its anticancer effects by inhibiting thymidylate synthase and incorporating its metabolites into RNA and DNA, thereby generating RNA and DNA damage (Longley et al., 2003). Despite the limited usage of 5-FU in various cancers due to the development of multi-drug resistance, it has been accepted as a first-line anti-cancer drug for HCC chemotherapy (Abdel-Hamid and Morsy, 2010; Patt et al., 2003; Porta et al., 1995; Stuart, 2012; Tetef et al., 1995; Tong et al., 2012; Uchibori et al., 2012).

In this study, treating HCC and colorectal cancer cell lines with 5-FU resulted in hnRNP K downregulation (Figure 3.6). This observation is consistent with a previous report that proposed hnRNP K as a predictive marker for response to 5-FU treatment in colorectal cancer (Zhang et al., 2010), but it was uncertain if this downregulation was an outcome of apoptosis. To eliminate the possibility that the reduction of hnRNP K was due to cleavage by caspases, cells were pre-treated with a pan-caspase inhibitor prior to the addition of 5-FU. The results revealed that hnRNP K downregulation was not due to cleavage by caspases, but rather, a direct consequence of 5-FU addition

(Figure 3.7). This observation however, does not eliminate the potential of hnRNP K to be cleaved by caspases, as hnRNP K has been suggested to be a potential caspase substrate by two large scale screening studies (Dix et al., 2008; Mahrus et al., 2008). In addition, hnRNP K has been reported to be downregulated by activated PKCδ in a proteasome-dependent manner (Gao et al., 2009) and has also been shown to be a proteolytic target of calpain (Kimura et al., 2003) as well as granzymes (van Domselaar et al., 2012). Thus, it remains to be ascertained if any of these proteins had a role to play in the reduction of hnRNP K following 5-FU treatment.

On the other hand, the experimental results have shown that 5-FU downregulates hnRNP K at the mRNA level (Figure 3.8), which would have played a major role in hnRNP K's reduction at the protein level. This reduction of hnRNP K mRNA expression by 5-FU could have been a direct effect of the drug itself, as 5-FU is known to inhibit RNA processing (Longley et al 2003). Alternatively, 5-FU-induced hnRNP K mRNA reduction could be mediated via other transcription factors such as c-Myc, as hnRNP K promoter region possesses its binding sequence (Carpenter et al., 2006). Indeed, 5-FU has been reported to reduce c-Myc mRNA expression level in a colorectal cancer cell line (Zhao et al., 2008a), indicating that reduced c-Myc following 5-FU treatment could have contributed to hnRNP K mRNA downregulation. However, as c-Myc is also a known downstream target of hnRNP K (Michelotti et al., 1996), it remains to ascertain whether the downregulation of c-Myc mRNA observed in the study by Zhao et al. (2008a) could have been a result of hnRNP K reduction instead.

5-FU was also demonstrated to reduce cell viability of HCC cells (Figure 3.4) via apoptosis (Figure 3.7), which is in agreement with previous studies (Ganten et al., 2004; Koike et al., 2006; Lasfer et al., 2006; Ma et al., 2011; Yi and Yang, 2003). In

addition, the experimental findings for both p53-null Hep 3B and HepG2 (p53+/+) (Figure 3.4 and Figure 3.7) are also consistent with previous reports that 5-FU causes apoptosis in both p53–dependent and –independent manners (Enge et al., 2009; Liu et al., 2006). Interestingly, this reduction of HCC cell viability by 5-FU in both cell lines with different p53 status correlated well with the demonstration that 5-FU-induced hnRNP K downregulation was independent of p53 (Figure 3.5 and 3.6).

Given that the downregulation of hnRNP K with siRNA decreased HCC cell viability (Figure 3.11) and induced apoptosis independently of p53 (Figure 3.18), it is compelling to propose hnRNP K downregulation as one of the contributors to the decrease in cell viability following 5-FU treatment. 5-FU has been previously demonstrated to increase caspase-8 activity in Hep 3B (Koike et al., 2006) and HepG2 cells (Yi and Yang, 2003), and can upregulate FASL death ligand at the transcriptional level in both Hep 3B and HepG2 cells (Eichhorst et al., 2000). This could provide an explanation for the marked decrease in Hep 3B and HepG2 cell viability 48 h post-5-FU treatment (Figure 3.4), where 5-FU-induced hnRNP K downregulation presumably decreased the expression levels of anti-apoptotic genes such as cFLIP and XIAP (Figure 4.11) while increased 5-FU-induced caspase-8 activation (Koike et al., 2006; Yi and Yang, 2003) accelerates apoptosis.

Furthermore, current findings might provide an explanation for the reported sensitization of TRAIL-resistant Hep 3B and HepG2 cells to TRAIL via the downregulation of cFLIP upon 5-FU addition (Ganten et al., 2004). It could be hypothesised that the reduction in hnRNP K induced by 5-FU (Figure 3.6) could have resulted in the corresponding downregulation of cFLIP at the transcriptional level (Figure 4.11 and Chen et al. (2010)), hence sensitizing the once TRAIL-resistant cells to TRAIL-mediated apoptosis.

Even though the downregulation of hnRNP K can contribute to p53-independent apoptosis during 5-FU treatment, the reduction of hnRNP K coupled with the induction of p53 during this treatment likely enhanced the pro-apoptotic effect observed. 5-FU is a known inducer of p53 (Ju et al., 2007), which can transactivate downstream cell cycle arrest and apoptotic targets that in turn activate the intrinsic apoptotic pathway (Longley et al., 2003). Thus, this explains the greater reduction in cell viability observed in HepG2 as compared to Hep 3B following 5-FU treatment (Figure 3.4).

Taken together, this current study has demonstrated for the first time that 5-FU treatment reduces hnRNP K expression level independently of p53 and that this reduction is not due to cleavage by caspases. Hence, the downregulation of hnRNP K by 5-FU can contribute to the decrease in cell viability observed during chemotherapeutic treatment.

### 5.4.2 hnRNP K reduction as an indicator of cell death

Apart from 5-FU, various chemotherapeutic agents such as mitomycin C, RITA, docetaxel and bicalutamide that can decrease cell survival have all been reported to downregulate hnRNP K (Barboro et al., 2009; Benelli et al., 2009; Enge et al., 2009; Rahman-Roblick et al., 2007). In contrast, various agents associated with proliferation or demonstrated to induce cell cycle arrest have been demonstrated to increase and stabilise hnRNP K levels, such as serum treatment, epidermal growth factor and heregulin-β1 that induce proliferation (Mandal et al., 2001; Ostrowski and Bomsztyk, 2003), as well as DNA damage treatments e.g. ionizing radiation, ultra-violet treatments and phleomycin that induce cell cycle arrest (Moumen et al., 2005). As such, it could be hypothesised that there is a positive correlation between the levels of

hnRNP K and cell survival, of which the decreased hnRNP K levels corresponding with decreased cell survival becomes particularly important during cancer treatment with chemotherapeutic drugs.

As illustrated in Table 5.1, the hnRNP K reduction following chemotherapeutic treatment could play a part in the induction of apoptosis, hence resulting in decreased cell viability as demonstrated in the treatment of HCC cells with 5-FU. Nevertheless, this decrease in hnRNP K could also occur simultaneously with the onset of apoptosis, e.g. various chemotherapeutic drugs treatments activated PKCδ, which downregulated hnRNP K in a proteasome-dependent manner and cleaved caspase-3 to bring about apoptosis (Gao et al., 2009). In addition, hnRNP K has been demonstrated to be a substrate for granzymes produced by cytotoxic T cells, which can also cleave caspase-3 to bring about apoptosis (van Domselaar et al., 2012). Apart from the two aforementioned scenarios, the decrease in hnRNP K might also be a result of the onset of cell death or apoptosis, as hnRNP K has also been reported to be a substrate of calpain and caspase (Dix et al., 2008; Kimura et al., 2003; Mahrus et al., 2008). Thus, the decrease in hnRNP K during chemotherapeutic treatment could function as a mechanism to induce apoptosis, be a simultaneous effect observed during apoptosis initiation or just a consequence of cell death.

**Table 5.1 Roles for hnRNP K reduction during chemotherapeutic therapy**

| Roles for hnRNP K reduction | Example | References |
|---|---|---|
| 1. Contribute to apoptosis induction | • 5-Fluorouracil | Current study |
| 2. A simultaneous effect observed during apoptosis | • Activated PKCδ downregulates hnRNP K and cleaves caspase-3 | Gao et al. (2009) |
| | • Both caspase-3 and hnRNP K are granzyme substrates | van Domselaar et al. (2012) |
| 3. A consequence of cell death | • A caspase substrate (Apoptosis) | Dix et al. (2008); Mahrus et al. (2008) |
| | • A calpain substrate (Necrosis) | Kimura et al. (2003) |

As this current study has demonstrated the anti-apoptotic function of hnRNP K in HCC cells with supporting evidence from other studies in various cancers, it can thus be inferred that the reduction in hnRNP K following treatment with various chemotherapeutic agents functions as a mechanism to remove barriers that resist cell death. In fact, hnRNP K was observed to be upregulated in neuroblastoma cells that are resistant to etoposide treatment (Urbani et al., 2005), further illustrating the importance of hnRNP K's anti-apoptotic role in preventing reduction in cell viability.

Overall, the decline in hnRNP K is a direct indication of reduced cell viability during various chemotherapeutic treatments, which can serve as a surrogate measure of these treatments' therapeutic effect. Thus, hnRNP K could potentially serve as a therapeutic marker of HCC treatment, indicating the response to chemotherapeutic treatment. This could also be extended to other cancers with elevated hnRNP K levels.

## 5.5 Conclusion

hnRNP K has been consistently reported to be upregulated in various cancers, including HCC that possesses apoptosis dysregulation as one of its hallmarks. While it has been previously reported that hnRNP K is implicated in apoptosis, its precise role in this process has never been clearly defined.

In this current study, the experimental findings have established the importance of hnRNP K in HCC cell survival, having demonstrated its contributions towards HCC's resistance to apoptosis. The role of hnRNP K in maintaining HCC cells' ability to inhibit apoptosis was revealed for the first time to be via the regulation of anti-apoptotic proteins that inhibits apoptosis induction at the caspase-8 and caspase-3/7 axis. It was also revealed that caspase-8 apoptotic signalling was pivotal for apoptosis induced in HCC cells following extended hnRNP K downregulation with siRNA.

Furthermore, given that the addition of a chemotherapeutic agent, 5-FU, to HCC cells downregulated hnRNP K independently of p53 and caspase cleavage, it is suggested that this reduction of hnRNP K by downregulation contributes to apoptosis induction in cancer cells.

Thus, the various findings in this study have shed light on hnRNP K's anti-apoptotic role in HCC, highlighting its potential as both prognostic and therapeutic markers in the management and surveillance of cancer progression in HCC patients.

# CHAPTER 6

## References

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# Appendices

# APPENDIX I: Media, Buffers and Solutions

## Media and buffers for cell culture use

### FBS (Fetal Bovine Serum)

Thaw 500 ml of FBS (Invitrogen) at 37°C water bath and heat inactivate at 56°C for 30min. Aliquot into 50 ml falcon tubes and store at -20°C till next use.

### DMEM culture medium with 10%FBS (DMEM/10%FBS)

| Component | Volume |
|---|---|
| DMEM (Invitrogen) | 450 ml |
| FBS (heat inactivated) | 50 ml |
| Total Volume | 500 ml |

Filter and store at 4°C till next use.

### Freezing medium

| Component | Volume |
|---|---|
| DMEM (Invitrogen) | 45 ml |
| FBS (heat inactivated) | 45 ml |
| DMSO | 10 ml |
| Total Volume | 100 ml |

Filter and store at 4°C till next use.

### PBS (Phosphate Buffered Saline) 10X

| Component | Weight (1L) |
|---|---|
| NaCl | 80g |
| KCl | 2g |
| $KH_2PO_4$ | 2.4g |
| $Na_{2\,h}PO_4$ | 14.4g |

Dissolve in 800 ml of Milli-Q water and adjust pH to 7.4 with HCl and top volume to 1L, autoclave and store at 4°C. Dilute to 1X solution before use.

## Buffers and solutions for molecular cloning

### TBE Buffer (Tris-Borate-EDTA) 10X

| Component | Weight (1L) |
|---|---|
| Tris base | 108g |
| Boric acid | 55g |
| EDTA | 9.3g |

Dissolve in 1L Milli-Q water, autoclave and store at room temperature.

### Luria Bertani Broth (LB Broth)

| Component | Weight (for 1L) | Final concentration (w/v) |
|---|---|---|
| Tryptone | 10g | 1% |
| Yeast Extract | 5g | 0.5% |
| NaCl | 10g | 1% |

Dissolve and top up with Milli-Q water to 1L, autoclave and store at 4°C.

### Luria Bertani Agar (LB Agar)

| Component | Weight (for 400 ml) | Final concentration (w/v) |
|---|---|---|
| Tryptone | 4g | 1% |
| Yeast Extract | 2g | 0.5% |
| NaCl | 4g | 1% |
| Agarose | 6g | 1.5% |

Dissolve and top up with Milli-Q water to 400 ml and autoclave. Cool to room temperature before the addition of antibiotics and pour into plates.

### Ampicillin

Dissolve ampicillin powder in Milli-Q water to obtain stock concentration of 200 mg/ml. Add appropriate amount into LB broth or agar to attain working concentration of 100 μg/ml.

## Buffers and Solutions for SDS-PAGE and Western Blot

### NP-40 Cell Lysis Buffer

| Component | Stock | Volume (100 ml) |
|---|---|---|
| 50mM Tris-HCl (pH7.8) | 1M Tris-HCl (pH7.8) | 5 ml |
| 150mM NaCl | 5M NaCl | 3 ml |
| 1% Nonidet P-40 | Nonidet P-40 (100%) | 1 ml |

Top up with Milli-Q water to 100 ml and store at 4°C till next use. Add in complete mini (Roche) and PMSF (working concentration 1mM) freshly before use.

### PMSF (phenylmethylsulfonyl fluoride)

Dissolve PMSF powder in isopropanol to yield concentration of 200mM and store at -20°C till next use. Ensure that the solids fully dissolve before use.

### Complete Mini (25X)

Dissolve 1 tablet (Roche) in 420 µl of RNase/ DNase free water to obtain 25X stock and store at -20°C till next use. Ensure that the solids fully dissolve before use.

### SDS- Polyacrylamide Gel Recipe

| | Separating gels | |
|---|---|---|
| | 10% | 12.5% |
| Component/ Volume | | |
| Water | 8.0 ml | 6.4 ml |
| 30% acrylamide mix (Bio-Rad) | 6.6 ml | 8.3 ml |
| 1.5M Tris (pH8.8) (Bio-Rad) | 5.0 ml | 5.0 ml |
| 10% SDS | 200 µl | 200 µl |
| 10% APS (Bio-Rad) | 100 µl | 100 µl |
| TEMED (Bio-Rad) | 20 µl | 20 µl |
| Total Volume (for 2 gels) | 20 ml | 20 ml |

Add in the order indicated, swirl well.

| | Stacking gels 5% |
|---|---|
| Component/ Volume | |
| Water | 2.84 ml |
| 30% acrylamide mix (Bio-Rad) | 0.83 ml |
| 0.5M Tris (pH6.8) (Bio-Rad) | 1.25 ml |
| 10% SDS | 50 µl |
| 10% APS (Bio-Rad) | 25 µl |
| TEMED (Bio-Rad) | 5 µl |
| Total Volume (for 2 gels) | 5 ml |

Add in the order indicated, swirl well.

**2X SDS-PAGE Sample Buffer**

| Component | Stock | Volume (100 ml) |
|---|---|---|
| 50mM Tris-HCl (pH7.8) | 1M Tris-HCl (pH7.8) | 12.5 ml |
| 20% Glycerol | 100% Glycerol | 20 ml |
| 4% β-mercaptoethanol | β-mercaptoethanol | 4 ml |
| 0.4% Bromophenol Blue (w/v) | Bromophenol Blue (Powder) | 0.4g |
| 4% SDS (w/v) | SDS (Powder) | 4g |

Top up with Milli-Q water to 100 ml and store aliquots in -20°C till next use.

**10X Tris-Glycine SDS-PAGE Running Buffer**

| Component | Weight (1L) | Final concentration (w/v) |
|---|---|---|
| Tris base | 30.2g | 25mM |
| Glycine | 144g | 192mM |

Top up with Milli-Q water to 1L. To obtain 1X buffer, add in 10% SDS to a final working concentration of 0.1% to 100 ml of 10X buffer and top up to 1L.

**10X Transfer Buffer**

| Component | Weight (1L) | Final concentration (w/v) |
|---|---|---|
| Tris base | 58.2g | 48mM |
| Glycine | 29.3g | 39mM |

Top up with Milli-Q water to 1L. To obtain 1X buffer, add in 3.75 ml of 10% SDS and 200 ml of Methanol to 100 ml of 10X buffer and top up with Milli-Q water to 1L.

**TBST Buffer**

| Component | Stock | Volume (1L) |
|---|---|---|
| 20mM Tris-HCl (pH7.6) | 1M Tris-HCl, pH7.6 | 20 ml |
| 150mM NaCl | 5M NaCl | 30 ml |
| 0.1% Tween-20 | Tween-20 | 1 ml |

Top up with Milli-Q water to 1L.

**Blocking Buffer**

Add in 5% non-fat milk to 1 × TBST buffer, store at 4°C

**Buffers and Solutions for Flow Cytometry Analysis**

**RNase A**

Dissolve RNase A (Sigma) in RNase/DNase free water (Invitrogen) to obtain 1 mg/ml stock. Aliquot and store at 4°C. Dilute 10X with RNase/DNase free water to 100 μg/ml working concentration before use

**PI (propidium iodide)**

Dissolve PI (Sigma) in RNase/DNase free water (Invitrogen) to obtain 2 mg/ml stock. Aliquot and store at 4°C in dark. Dilute 40X with RNase/DNase free water to 50 μg/ml working concentration before use

## Buffers and Solutions for Immunofluorescence

### PFA (paraformaldehyde, 4% in PBS) (freshly prepared)

Dissolve 0.4g paraformaldehyde in ~8 ml PBS, warm at 56°C water bath. Add 10N NaCl drop wise until solution clears. Top up to 10 ml with PBS

### Triton-X100 (0.1% in PBS)

Add 1 ml Triton-X100 (Bio-Rad) to 99 ml PBS to prepare 1% stock. 10 × dilution with PBS to make 0.1% Triton-X100 before use

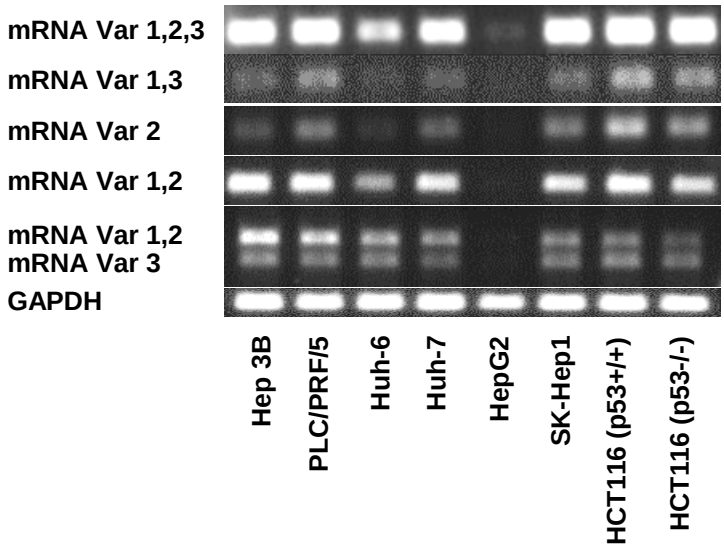
### BSA (bovine serum albumin, 5% in PBS) (freshly prepared)

Dissolve 0.5g BSA in 10 ml PBS before use.

### PBST

To 1L of 1X PBS, add in 1 ml of Tween-20.

# APPENDIX II: Supplementary Results




**Appendix II Figure 1. Low hnRNP K mRNA levels in HepG2**
Semi-quantitative RT-PCR results of hnRNP K mRNA levels in the various cell lines. PCR primers targeting different regions of hnRNP K detect for common regions found in the different alternatively spliced mRNA variants (1 to 3). hnRNP K mRNA levels remained consistently low in HepG2 even with the use of primers targeting different regions of hnRNP K, ruling out possible mutation(s) in the coding region of hnRNP K in this cell line.

# APPENDIX III: Publications

hnRNP K suppresses apoptosis independent of p53 status by maintaining high levels of endogenous caspase inhibitors
**Xiao Z**, Ko HL, Goh EH, Wang B, and Ren EC.
Carcinogenesis- *Article in press*

The p53 response element and transcriptional repression
Wang B, **Xiao Z**, Ko HL and Ren EC.
Cell Cycle. 2010; 9(5):870-9.

Redefining the p53 response element
Wang B**, Xiao Z** and Ren EC.
Proc Natl Acad Sci U S A. 2009;106(34):14373-8.

LIM and SH3 protein 1 (Lasp1) is a novel p53 transcriptional target involved in hepatocellular carcinoma
Wang B, Feng P, **Xiao Z**, Ren EC.
J Hepatol. 2009; 50(3):528-37.

15th International p53 workshop, 2010
Poster presentation:
A dinucleotide core within the p53 response element is critical in controlling p53-dependent transcriptional regulation
**Xiao Z.**, Wang B., Liu J., Ren E.C.

2009 International Meeting- The Molecular Biology of Hepatitis B Viruses
30 Aug 2009 - 2 Sep 2009
Poster presentation:
The LIM and SH3 Protein 1 (LASP1) is a novel p53 transcriptional target involved in HBV-associated hepatocellular carcinoma
**Xiao Z.**, Ren E.C., Wang B.