SCREENING FOR NOVEL PROTEIN-PROTEIN INTERACTIONS IN LIVING HUMAN CELLS WITH THE HELP OF THE SPLIT-UBIQUITIN SYSTEM

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NATIONAL UNIVERSITY OF SINGAPORE

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Summary

Human Suppressor of RNA polymerase B (hSRB7) /Med21 is a component of the mammalian Mediator complex, and this subunit has been shown to interact directly with human S-phase kinase associated protein 1 (hSkp1). The latter is a component of the hSkp1-Cul1-F-box protein (SCF) complex, an E3 ubiquitin ligase involved in the process of ubiquitylation of target proteins.

In this study, the protein-protein interaction between hSkp1p and hSrb7p was shown using the yeast-split ubiquitin system and mammalian split-ubiquitin system, respectively. As it is speculated that hSkp1p may be recruited to the holoenzyme by its interaction with hSrb7p, the biological relevance of this interaction and the role of hSkp1p in transcription were examined. Small-interfering RNA was used to reduce hSkp1 transcripts in HeLa cells. This was coupled to reverse-transcription polymerase chain reaction (RT-PCR) to study the efficiency of the induction of Hsp70B' mRNA and to examine the effects of hSkp1 on activated transcription. The results indicated that hSkp1p is necessary for the full activated transcription of Hsp70B' by heat-shock. Future experiments would include chromatin-immunoprecipitation assay (Ch-IP) to show that hSkp1p is indeed recruited to the heat-shock promoter region together with hSrb7p.

In the last part of this project, the yeast split-ubiquitin system, a protein-protein interaction assay that is based upon a fragment complementation coupled to a conditional proteolysis strategy, was used to isolate interacting partners of hSkp1p in hope of detecting novel roles of hSkp1p in cellular processes. Complementary DNA (cDNA) isolated from HeLa cells were fused to the N-terminus of ubiquitin (N_{ub}-cDNA) in order to create a cDNA library for screening of interacting proteins in *S. cerevisiae*. The bait

was hSkp1p fused to the C-terminal half of ubiquitin (C_{ub}) followed by orotidine-5'-phosphate decarboxylase (Ura3) that had been modified such that the first amino acid was replaced by an arginine residue (RUra3), resulting in the fusion protein hSkp1-C_{ub}-RUra3. Cyclin-dependent kinase (Cdk) 2-interacting protein (CINP) was isolated in the screen; this protein is associated with active cyclin E/Cdk2 and cyclin A/Cdk2 complexes, and has been suggested to be involved in DNA replication. Therefore this could indicate that hSkp1p may play a role in DNA replication.

This study has provided evidence of hSkp1p in transcription, and strengthened the link between ubiquitin-proteasome pathway and transcriptional regulation. Future work on finding new interacting partners of hSkp1p and characterizing their interaction would allow us to understand the various cellular processes that hSkp1p, and possibly the ubiquitin-proteasome pathway, may be involved in.

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LIST OF ABBREVIATIONS

5-FOA	5-fluoroorатic acid
6-TG	6-thioguanine
C_{ub}	C-terminal half of ubiquitin
DMEM	Dulbecoo's modified Eagle's medium
dNTP	Deoxyribonucleotide tri-phosphate
FCS	Fetal calf serum
Gpt2	Guanine phosphoryltransferase 2
HA	Haemagglutinin
HAT	Hyproxanthine/aminopterin/thymidine
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
LB	Luria Bertani
N_{ub}	N-terminal half of ubiquitin
ORF	Open reading frame
PCR	Polymerase chain reaction
PIC	Preinitiation complex
RGpt2	Guanine phosphoryltransferase 2 reporter modified to begin with an arginine residue
RNAPII	RNA polymerase II
RT-PCR	Reverse-transcription polymerase chain reaction
RUra3	Orotidine-5'phosphate decarboxylase reporter modified to begin with an arginine residue
SAGA	Spt-Ada-Gcn5-acetyltransferase
SDS	Sodium dodecyle sulfate
siRNA	Small-interfering RNA
Skp1	S-phase kinase associated protein 1
Srb	Suppressor of RNA polymerase B
TAD	Transcriptional activation domains
TBP	TATA-box binding protein
Ubps	Ubiquitin-specific proteases
Ura3	Orotidine-5'phosphate decarboxylase

CHAPTER 1

INTRODUCTION

51. Introduction

The Mediator complex is involved in transcriptional regulation, transducing signals from activators and repressors to RNA polymerase II (Myers and Kornberg, 2000). It was first discovered in *Saccharomyces cerevisiae* as an activity that could mediate and integrate the signals of transcriptional activators to the basal RNA polymerase machinery, and was therefore termed Mediator (Kelleher *et al.* 1990). Subsequently, biochemical and structural studies in various laboratories revealed a number of mammalian Mediator complexes like the thyroid receptor associated proteins (TRAP) (Fondell *et al*., 1996), SRB/MED-containing cofactor complex (SMCC) (Gu *et al*., 1999). Today, it is established that the mammalian Mediator complex is made up of at least 30 subunits (Sato *et al*. 2004), although it should be noted that some subunits are missing in some purifications performed. This may be due to the different techniques used that gave rise to the discrepancies, or because there are different Mediator entities in the cells, each with different functions (Myers and Kornberg, 2000; Näär *et al*., 2001 and Sato *et al*., 2004).

One subunit found in both budding yeast and mammalian Mediator complex is the Suppressor of RNA polymerase B (SRB7) / MED21. This is an essential gene in yeast, and it was identified in a suppressor screen for mutants that restore the viability of cells with truncations in RNA polymerase II CTD (Hengartner *et al*., 1995). Later on, it was shown that ySrb7p interacted with repressor yTup1p both *in vitro* and *in vivo* (Gromöller and Lehming, 2000). The proposed model was that yMed6p, another subunit in the Mediator complex, and yTup1p both compete for binding to ySrb7p; collectively, this

indicated a direct effect of yTup1p mediated repression on the holoenzyme. SRB7 is an essential gene for the very early stages of embryogenesis in mouse (Tudor *et al*., 1999).

Using the yeast split-ubiquitin system in *S. cerevisiae*, Debra Morley discovered that hSrb7p interacts with hSkp1p (PhD Thesis dissertation, Liverpool John Moores University, 2003). The yeast split-ubiquitin system is based on the conditional degradation design (Wittke *et al*., 1999). The reporter protein used was orotidine-5'-phosphate decarboxylase (Ura3) that had been modified such that the first amino acid was now an arginine residue (RUra3). One protein was fused to the N-terminus of ubiquitin (N_{ub}) and the second protein was fused to the C-terminus of ubiquitin (C_{ub}) that was extended by the RUra3 protein (C_{ub}-RUra3). According to the N-end rule, which states that the N-terminal amino acid mediates the *in vivo* half-life of a protein (Varshavsky, 1996), arginine is a destablishing residue in *S. cerevisiae*. A really-interesting-new-gene (RING) finger-containing E3 ubiquitin ligase, Ubr1p, recognizes the N-terminal residues and adds a chain of multi-ubiquitin to the internal lysine residue of the substrate (Dohmen *et al.,* 1991). Subsequently, the poly-ubiquitylated substrate is degraded by the 26S proteasome. If the two proteins interact in the cell, the two halves of ubiquitin would be in close proximity and a native-like ubiquitin moiety would be formed. This would cause the release of the reporter protein by ubiquitin-specific proteases (Ubps), and its degradation by the enzymes of the N-end rule. As a result, the reporter protein RUra3 was degraded and the phenotype of the yeast cells became uracil-deficient. Such protein-protein interactions were detected by the use of media containing 5-fluoroorotic acid (5-FOA), a drug which counterselects the Ura3 enzymatic activity. Using this protein-protein interaction assay and hSrb7-C_{ub}-RUra3 as bait, Debra Morley

had isolated a C-terminal portion of hSkp1p fused downstream of N_{ub} using a N_{ub}-cDNA fusion library. The interaction between hSkp1p and hSrb7p was also observed in the human cells using the mammalian split-ubiquitin system. This assay is similar to the yeast-split ubiquitin in that it is also a fragment complementation assay based on a conditional proteolysis strategy, but the reporter gene used differs from the yeast system. Rojo-Niersbach *et al*. (2000) had shown the use of an RGpt2 reporter protein in selection of protein interactions in the human fibroblast cell line HT1080HPRT$^-$ (Pellegrini *et al*., 1989). This cell line lacks the enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT), and it is therefore sensitive to medium containing hypoxanthine/aminopterin/thymine (HAT), but is resistant to medium containing 6-thioguanine (6-TG). The guanine phosphoryltransferase 2 (gpt2) gene of *Escherichia coli* is able to complement the HPRT$^-$ deficiency. Should hSkp1p and hSrb7p interact *in vivo*, the two proteins would be brought in close proximities. This resulted in the reconstitution of ubiquitin, and Ubps would cleave the hSrb7-C_{ub}-RGpt2 fusion, and the RGpt2 reporter protein would be rapidly degraded by the enzymes of the N-end rule. Therefore cells that had co-expressed the interacting protein pair hSrb7-C_{ub}-RGpt2 and N_{ub}-hSkp1 would be HAT sensitive and 6-TG resistant. In addition, Debra Morley had shown that the interaction was a direct one by performing pull-down with glutathione-S-transferase (GST)-tagged proteins.

S-phase kinase protein 1 (Skp1) had been discovered due to its association with cyclinA-Cdk2 complex (Zhang *et al*., 1995). However it was later established by Bai *et al*. (1996) that Skp1p does not interact directly with cyclinA-Cdk2 complex but through another protein, the S-phase kinase associated protein (Skp2). In *Saccharomyces*

cerevisiae, Skp1p was found in a protein-protein interaction screen for interacting partners of cyclin F, and protein analysis of cyclin F revealed it had a novel motif (Bai *et al.,* 1996). This motif was termed the F-box domain, and the family of F-box proteins was born. A year later, the E3 ubiquitin ligase complex responsible for ubiquitylation of target proteins was discovered, and this complex was termed 'Skp1-Cullin1-F-box protein' or SCF (Skowyra *et al*., 1997 and Feldman *et al*., 1997). This complex is part of the ubiquitin-proteasome pathway, another important pathway in the cell to ensure the turn-over of proteins by degrading them when they are no longer necessary. It starts with the process of ubiquitylation, defined as the covalent attachment of ubiquitin to the target protein (Welchman *et al*., 2005). Essentially, an isopeptide bond is formed between a ε-amino group of a lysyl residue in a target protein and the C-terminal glycine of ubiquitin. Three sets of enzymes are needed for ubiquitylation, and they are the ubiquitin-activating (E1), ubiquitin-conjugating (E2) and the ubiquitin ligase (E3). The conjugation of a chain of ubiquitin marks the substrate protein to be degraded by the 26S proteasome, an ATP-dependent multisubunit protease (Welchman *et al*., 2005). In the SCF complex, the F-box protein is the variable component in the complex, and some 67 proteins that have contain the F-box sequence motif had been discovered to date (Jin *et al.,* 2004). Interestingly, Skp1p has been found to interact with several proteins that do not contain the F-box motif. For instance, the interaction of ySkp1p with Ctf13p and Sgt1p in yeast is important for the formation of kinetochore complex, CBF3 (Kaplan *et al.,* 1997 and Rodrigo-Brenni *et al*., 2004). ySkp1p is also known to interact with Rav1p and Rav2p, and together these proteins form a complex known as 'regulator of the H^+ ATPase of the vacuolar and endosomal membranes' (RAVE) (Seol *et al.,* 2001).

Incidentally, SKP1 was found to have significant homology to eukaryotic transcription factor Transcription Elongation Factor B (TCEB1) (Sowden *et al.* 1995), and TCEB1 is now more commonly known as ElonginC, a subunit of the transcription factor SIII elongation complex (Bradsher *et al*., 1993a,b). This complex had been isolated as a stimulatory factor of RNA polymerase II during elongation by stimulating the rate of transcription, although the exact mechanisms have not been determined to date (Sims *et al*., 2004). In addition, ElonginC is able to form a complex with ElonginB, Cul2p and the RING-protein Hrt1p to form a multiple subunit E3 ubiquitin ligase (Lonergan *et al*., 1998 and Iwai *et al*., 1999). The ElonginBC-Cul2p E3 ligase binds to various proteins that carry the BC-box motifs like von Hippel-Lindau (VHL) tumor suppressor (Wykoff *et al*., 2001), and such BC-box proteins are similar to the F-box proteins in the SCF complexes in that they act as an adapter between the substrate and the ubiquitin ligase. Interestingly, Brower *et al*. (2002) had shown that the Elongin BC-Cul2p complex is able to bind to Med8p, a component of the Mediator complex, and reconstitute ubiquitin ligase activity was detected.

Given the interactions of hMed8p and hSrb7p with hElonginC and hSkp1p respectively (Figure 1), one may speculate that the ubiquitin ligase activity is recruited to the holoenzyme or the Mediator complex at the promoter region. This could be the link between ubiquitin-proteasome pathway and transcription. According to the model by Muratani and Tansey (2003), ubiquitin ligase could be recruited to the site of transcription and ubiquitylate many factors like the activators, histones or even the RNA polymerase II. These target proteins, like the activator, would then be degraded by the 26S proteasome, thereby promoting transcriptional elongation. However, more data is

necessary to understand the link between ubiquitin-proteasome pathway and transcription.

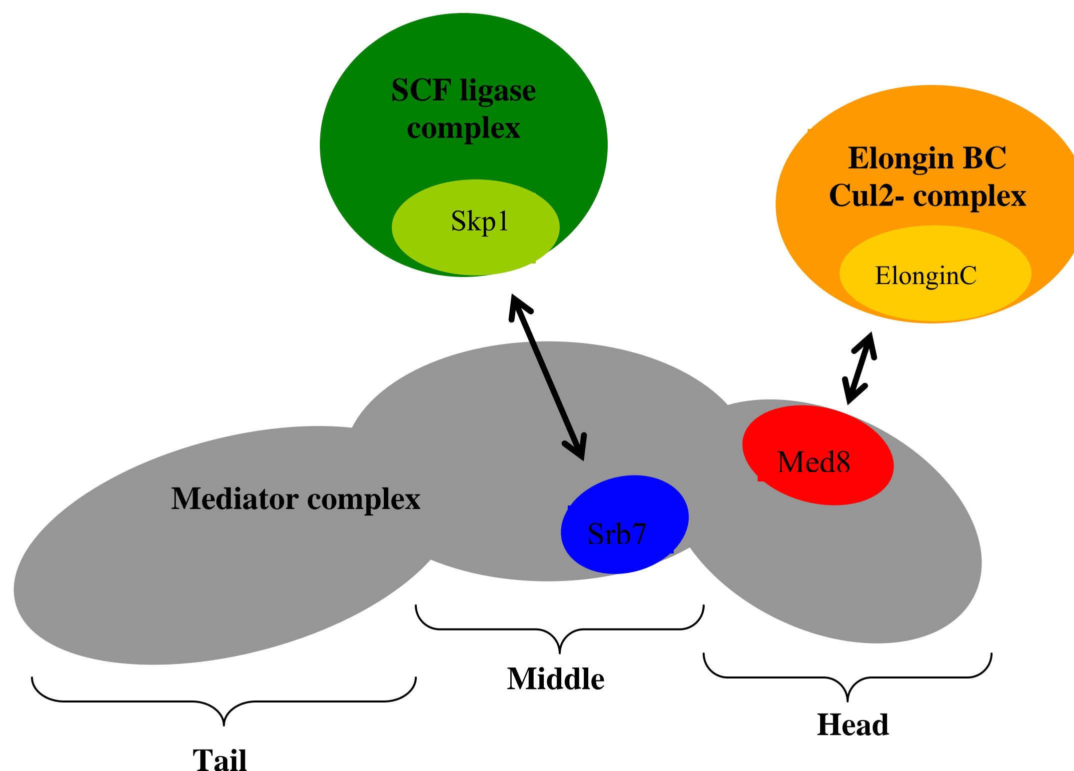

Figure 1: Schematic representation of the interactions between Mediator subunits hSrb7p and Med8p with Skp1p and ElonginC.
The Mediator complex (represented in grey) is divided into three structural domains, the head, middle and tail domains; hSrb7p (shown in blue) is believed to be in the middle domain, whereas hMed8p (shown in red) is proposed to be in the head domain. hSkp1p is associated with the E3 ubiquitin ligase SCF complex, whereas Elongin C is part of the E3 ubiquitin ligase Elongin BC complex. The arrows indicate the protein-protein interactions.

Skp1p may have a much wider role than just as an adapter protein in the SCF E3 ubiquitin ligase, given the fact that it can bind to proteins that do not have an F-box motif, and that it is homologous to Elongin C. In the first part of this study, the yeast-split-ubiquitin system and the mammalian ubiquitin system was used to verify that full-length hSkp1p and hSrb7p indeed interact in *S. cerevisiae* and human cells respectively. Now that Skp1p has been shown to bind to hSrb7p, one aim of this project was to

examine the biological relevance of the interaction. It is plausible that hSkp1p is recruited to the holoenzyme by hSrb7p, and therefore the next step would be to understand the effects of hSkp1p on transcription. To do this, RNA interference was employed to reduce hSkp1 transcripts in HeLa cells, and the effects of this reduction was examined on the heat-shock gene transcription using reverse-transcription polymerase chain reaction (RT-PCR). If hSkp1p played an important role in transcription, for instance, in transcriptional activation, the knock-down of hSkp1 would reduce the activated Hsp70B' transcripts. In addition, protein-protein interaction assay to search for interacting partners of hSkp1p may give us a better understanding of the functions of hSkp1p. Thus the second aim of this project was to use the yeast split-ubiquitin system to screen for interacting partners of hSkp1p fused to C-terminus of ubiquitin (C_{ub}) followed by the Ura3 enzyme that had been modified to begin with an arginine residue (hSkp1-C_{ub}-RUra3). Complementary DNA (cDNA) isolated from HeLa cells were fused to N-terminus of ubiquitin (N_{ub}-cDNA), thereby creating a cDNA library which was used to screen for interacting partners of hSkp1p.

This study provides an insight into the biological relevance of the protein-protein interaction between hSkp1p and hSrb7p, and allows one to examine the role of hSkp1p in transcription. These results may provide evidence to strengthen the link between ubiquitin-proteasome pathways and transcription regulation, and new regulatory strategies of transcription involving the ubiquitin-proteasome pathway may be uncovered to allow us to understand the underlying mechanisms of transcriptional regulation. Furthermore, the characterization of proteins that interact with hSkp1p may help us

discover the cellular processes that hSkp1p, and possibly the ubiquitin-proteasome pathway, may be involved in.

CHAPTER 2

SURVEY OF LITERATURE

2. SURVEY OF LITERATURE

2.1 Transcription of eukaryotic protein-coding genes

Eukaryotes have many genes along their chromosome, and many studies have been done to elucidate how these genes are expressed and regulated. It was discovered that the purified RNA polymerase were insufficient in initiating transcription *in vitro* (Sentenac, 1985). Thus it was clear that additional factors were necessary for selective transcription initiation. Following this, general transcription factors (GTFs) were identified through fractionation of cell extracts (Zawel and Reinberg, 1992; Conaway and Conaway, 1993). These factors permit efficient selective initiation by RNA polymerase II (RNAPII). The set of basal or GTFs required for specific promoter binding by RNA polymerase II *in vitro* includes TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH.

A second class of transcription factors that was identified are the activator proteins that are capable of binding to DNA sequences either upstream or downstream of the promoters. These activators are able to stimulate the initiation of transcription, and so it was thought that these eukaryotic proteins were like their prokaryotic counterparts in that these proteins would exert their effect directly through the transcription machinery (Myers and Kornberg, 2000). This was proven otherwise with the discovery of a third class of transcription factors: the transcriptional coactivator complexes (Bjorklund *et al*., 1999; Näär *et al*., 2001). These proteins act as adaptors between activators (or repressors) and the transcription machinery, and two main groups of such regulators can be differentiated. One group affects the chromatin template, and the other group consists of regulators that require RNA polymerase II and its associated

proteins to function. The first group includes chromatin modifying complexes, histone acetyltransferase complexes and histone deacetylase complexes. The second group is made up of TBP-associated factors (TAFs), universal stimulatory activity (USA) and Mediator complexes. The latter will be discussed below.

2.1.1 Discovery of the Mediator complex

The observation of how one transcriptional activator may interfere with the effects of another in yeast (Gill and Ptashne, 1988) was one of the first indirect evidence for the presence of an activity which acts as a common target for transcriptional activators. This phenomenon of activator interference was termed squelching. At that time, it was thought that the general transcription factors like TFIIB and RNA polymerase II may be the source of this interference as Brandl *et al.* (1989) and Stringer *et al.* (1990) have shown that these proteins bind to activator proteins. However, the work done by Kelleher *et al.* (1990) proved otherwise. When activator interference was reproduced *in vitro*, the addition of RNA polymerase II or the various general transcription factors in excess amounts did not reverse the interference. This suggested that the target of the activators is distinct from the basal transcriptional machinery. Instead, it was the addition of a partially purified yeast component that lifted the interference. The activity in this component was believed to mediate and integrate the signals of transcriptional activators on the basal RNA polymerase machinery, and was termed Mediator. Further studies carried out by Flanagan *et al*. (1991) presented a direct evidence for a mediator that caused the *in vitro* stimulation of transcription by activators Gal4-VP16 and Gcn4.

The other concurrent line of work was in the analysis of yeast genetics, and was initially unrelated. In the screen for suppressors of partial truncations in the C-terminal domain (CTD) of the large subunit of RNAPII, a suppressor of RNA polymerase B (SRB) protein termed Srb2 (Med20) was isolated as extragenic suppressors of the cold-sensitive phenotype of CTD domain deletion mutants (Nonet and Young, 1989). Subsequent work by Thompson *et al*. (1993) lead to the identification of three other SRB proteins – Srb4 (Med17), Srb5 (Med18) and Srb6 (Med22), and it was shown that these four SRB proteins are components of a multisubunit complex that can bind to recombinant CTD protein. It was the purification of the Mediator activity that converged the two lines of work together. The Mediator was shown to be made up of some 20 polypeptides, and a holoenzyme form of RNAPII, which consisted of the mediator associated with core 12-subunit of the polymerase was isolated (Kim *et al*., 1994; Koleske and Young, 1994).

The existence of the Mediator complex in higher eukaryotes was not immediately found, and it was thought that there was no conservation of the complex during the evolution of the higher organisms. Subsequently, biochemical and structural studies in the various labs revealed a number of mammalian Mediator complexes; the list includes thyroid receptor associated proteins (TRAP) (Fondell *et al*., 1996), SRB/MED-containing cofactor complex (SMCC) (Gu *et al*., 1999), a vitamin D receptor (VDR)-interacting complex (DRIP) (Rachez *et al*., 1999), an SREBP-interacting complex (ARC) (Näär *et al*., 1998), an E1A-interacting complex (human Mediator) (Boyer *et al*., 1999), the upstream stimulatory activity (USA)- derived positive cofactor 2 (PC2) (Malik *et al*., 2000) and cofactor required for Sp1 (CRSP)

complex (Ryu *et al*., 1999), the negative regulator of activated transcription (NAT) (Sun *et al*., 1998), and the mouse Mediator complexes (Jiang *et al*., 1998). The basis for the isolation of these complexes can be divided into three groups. In the first group, these complexes were isolated on their ability to interact with specific activators. For DRIP, it was through ligand-bound vitamin-D receptor, ARC through sterol-response-element-binding protein (SREBP) and VP16, TRAP complex through its association with ligand-bound thyroid-hormone receptor, and human Mediator through E1A. As for the second group, the isolation had been based on homology of specific subunits to the yeast Mediator polypeptides. SMCC, NAT and the murine mediator fall into this group. PC2 and CRSP make up the third group which was isolated on the basis of its coactivator activities; CRSP as a coactivator for Sp1 (Ryu *et al*., 1999), and PC2 for several activators like Sp1 and GAL4-based activators (Kretzschmar *et al*., 1994).

The proteins that make up the mammalian Mediator are not as defined as the yeast Mediator. For instance, the mammalian Mediator subunits like TRAP220 (MED1), Rgr1 (MED14), TRAP80 (MED17), TRAP36 (MED4), MED6, MED7, and Srb7 (MED21), are consistently found in nearly all Mediator purifications. However, for other Mediator proteins like the TRAP25 (MED30), MED8, CRSP70 (MED26), and ARC92 (MED25), these have been identified in only a few preparations (Sato *et al*., 2004). This could be due to the different purification techniques used; some techniques may not be sufficiently sensitive or that there were protein loss during the purifications. A more intriguing explanation would be that the differences could be due to the different entities of the Mediator complexes under various conditions, an indicator of the different functions the complex has (Conaway *et al*., 2005). In a bid to define the

subunit composition of the mammalian Mediator, Sato *et al*. (2004) used the multidimensional protein identification technology (MudPIT) approach. A total of six different Mediator subunits were FLAG-tagged for the isolation of their associated proteins, and all 30 of the subunits previously identified as subunits of mammalian Mediator-like complexes were isolated. Additional proteins that were isolated with the epitope tagged subunits were the alternative forms of the kinase module subunits Srb8 (Med12), Srb9 (Med13) and CDK8. A unified nomenclature for the subunits of the Mediator complexes has recently been suggested to facilitate in cross-species comparisons (Bourbon *et al*., 2004).

2.1.2 Role of Mediator in transcription

The Mediator complex is known to interact with the CTD of the RNA polymerase II, specifically with the unphosphorylated form of CTD (Myers *et al*., 1998). The unphosphorylated form of the polymerase II is known to be an indication of promoter-bound initiation complex, whereas the phosphorylated form is an indication of RNAPII engaging in active elongation. It has been assumed that the role of Mediator is to nucleate preinitiation complex (PIC) consisting of the RNAPII and GTFs to form an active transcribing complex. Interestingly, Takagi *et al*. (2005) reported that the most abundant form of the Mediator is its free form, and not as a holoenzyme complex. This has implications that the Mediator and the RNAPII are recruited independently to the promoter region to form the PIC. The finding is supported by several works like that of Park *et al*. (2001) where instead of the holoenzyme, the Mediator is rapidly recruited to the heat shock promoter by heat shock factor (HSF) upon heat shock in *Drosophila*

melanogaster. Therefore, the model proposed for transcription regulation by the Mediator dictates that a DNA-binding activator brings the Mediator to the promoter, and a "scaffold platform", which includes GTFs like the TFIIB and TFIID, is established for the recruitment of RNAPII. As RNAPII escapes the promoter clearance and moves on to the elongation stage, the platform remains at the promoter to enable the rapid assembly of a new round of transcription known as reinitiation (Yudkovsky *et al*., 2000; Chadick and Asturias, 2005).

Specific interactions have been demonstrated between DNA-binding transcriptional activators and subunits of the Mediator via the transcriptional activation domains (TADs) of the former. For instance, the mammalian Rgr1 (Med14) interacts with activator STAT2 and therefore indicates that the Mediator is involved in interferon activated gene regulation (Lau *et al*., 2003). In addition, Trap100 (Med23) is able to bind to activators like the adenovirus E1A protein (Boyer et al., 1999), and Med25 can bind to the TAD of VP16 (Mittler *et al*., 2003). Therefore, these interactions strongly suggest that the Mediator activates transcription and is recruited to the promoter region for the formation of the pre-initiation complex. The role of Mediator in transcription repression has also been reported, especially for the Srb8-11 module (Song *et al*., 1998; Lee *et al*., 2000 and Kim *et al*., 2004)

There are also a few enzymatic activities associated with the Mediator complex known to date. Lorch *et al*. (2000) showed that purified Mediator has histone acetyltransferase (HAT) activity, and has direct interactions with free nucleosomes. This HAT activity has been attributed to the Nut 1 subunit. However, as NUT1 is a non-essential gene in yeast, it is believed that the HAT activity would not be a major role in

function of the Mediator. Kinase activity has also been associated with the Mediator complex, especially for subunits Cdk8-CyclinC. In *S. cerevisiae*, this kinase has been shown to be the enzyme responsible for the phosphorylation of transcriptional activator Gcn4, and this modified form of Gcn4 was subsequently poly-ubiquitylated and degraded (Chi *et al*., 2001). The cyclin H subunit of TFIIH is another target of cdk8-cyclinC; the phosphorylation of cyclin H results in the repression of TFIIH CTD kinase, therefore repressing the role of TFIIH in transcription (Akoulitchev *et al*., 2000). There were also suggestions that an ubiquitin ligase activity could be recruited to the Mediator complex as seen for the Med8p subunit interacting with Elongin BC proteins (Brower *et al*., 2002). The group had shown that together with ubiquitin ligase components Cul2p, Rbx1p, Elongin B and Elongin C, Med8p was able to reconstitute an E3 ubiquitin ligase.

2.1.3 Structure of the Mediator complex

Purification of the yeast Mediator complex showed that it was a large complex of approximately 1MDa, and single particle electron microscopy (EM) was employed for characterization of the structure of the complex. Isolated Mediator in its free form has been observed to be a compact structure. Upon incubation with the polymerase, the Mediator undergoes conformational changes and becomes an extended structure, with three distinctive Mediator domains that envelope the globular polymerase. These domains are characterized as head (h), middle (m), and tail (t) (Asturias *et al*., 1999; Dotson *et al*., 2000). According to the proposed locations of the core Mediator modules, the tail region is believed to correspond to the Gal11 module, the Med9/10 module to

the middle domain and the Srb4 module to the head domain (Figure 2; Boude *et al*., 2002).

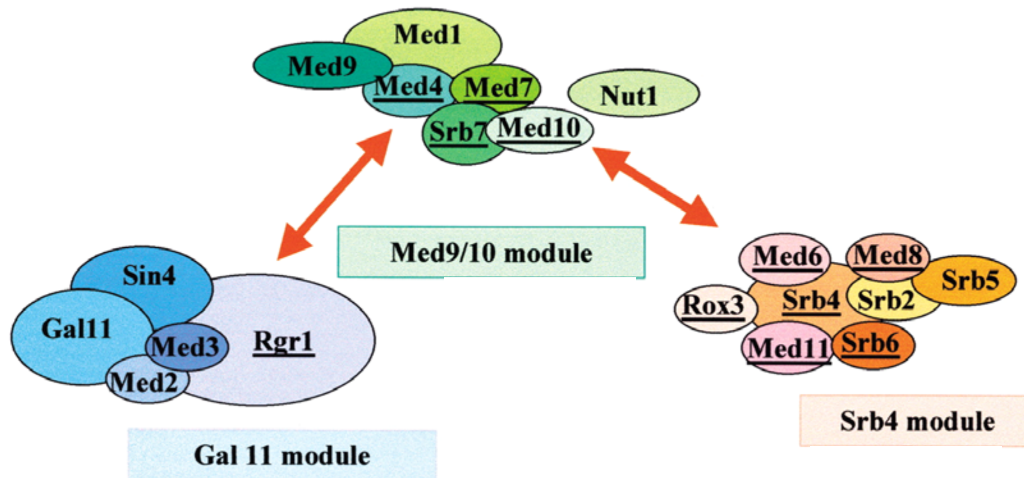

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Figure 2. Structural organization of the *S. cerevisiae* core Mediator associated with RNA polymerase II. Reprinted from Cell, 110(2), Boube *et al*., Evidence for a mediator of RNA polymerase II transcription regulation conserved from yeast to man, 143-51; Copyright (2002) with permission from Elsevier.
The figure shows the proposed locations of core Mediator modules and a model for subunit organization based on genetic, biochemical, and two-hybrid interactions (Boube *et al*., 2002; Ito *et al*., 2001). Underlined subunits are essential for viability.

2.1.4 Suppressor of RNA polymerase B (SRB7) / Med21

This is an essential yeast gene, and the identification of this gene was through a screen for a recessive mutation that restores the viability of mutants with truncations in RNA polymerase II CTD (Hengartner *et al*., 1995). Chao *et al*. (1996) identified sequences similar to yeast Srb7 through EST databases, and the predicted 144-amino acid human protein is 35% identical to the yeast Srb7 protein. Mouse knockout studies have shown that the SRB7 subunit of TRAP/SMCC is essential for the very early stage of embryogenesis (Tudor *et al*., 1999). Gromöller and Lehming (2000) showed that yeast Srb7p interacted with repressor Tup1p both *in vitro* and *in vivo*. The model

proposed was that Med6p, another subunit in the Mediator complex, and Tup1p both compete for binding to Srb7p; collectively, this indicated a direct effect of Tup1p mediated repression on the holoenzyme.

Section 2.2 Insight into Skp1 and the SCF ligase

2.2.1 Ubiquitin and the process of ubiquitylation

Ubiquitin is a small protein of 76 amino acids, and yet when multiple moieties are covalently attached to a target protein, this modification can lead to the degradation of that protein via the 26S proteasome. Hence ubiquitin has earned its name as the "kiss-of-death" protein. The investigations into the chemistries and the functions of protein degradation have lead to three scientists, Avram Hershko, Aaron Ciechanover, and Irwin Rose, receiving the Nobel Prize in Chemistry in 2004 (Giles, 2004). Today, much more information is available about ubiquitin since its discovery in 1980 as an ATP-dependent proteolysis factor (Wilkinson *et al.*, 1980); recent findings also indicate that the modification of proteins with ubiquitin has a wider role than simply a degradation signal.

Ubiquitin is absent in prokaryotes, but some homologues of the E1 ubiquitin-activating enzymes have been discovered (Welchman *et al.*, 2005). Ubiquitylation is defined as the covalent attachment of ubiquitin to the target protein. Essentially, an isopeptide bond is formed between a ε-amino group of a lysyl residue in a target protein and the C-terminal glycine of ubiquitin. Three sets of enzymes are needed for ubiquitylation, and they are the ubiquitin-activating (E1), ubiquitin-conjugating (E2) and the ubiquitin ligase (E3). This pathway is summarized in Figure 3.

Two different classes of modification with ubiquitin can occur: monoubiquitylation and polyubiquitylation. Monoubiquitylation, as its name implies, involves the transfer of only a single ubiquitin residue onto a lysine or several lysines of the substrate protein. This form of modification is now known to regulate the localization and activity of the target protein; the three main cellular processes that monoubiquitylation is involved in are the regulation of histones, endocytosis (Dupre *et al*., 2004), and budding of retrovirus (Hicke, 2001). Although monoubiquitylation is the first step to polyubiquitylation, the conjugation of a single ubiquitin molecule to the protein does not result in its degradation. As for polyubiquitylation, several ubiquitins are added to a single lysine residue of the target protein. This chain of ubiquitin is usually formed through the isopeptide bond between the C-terminal glycine of ubiquitin and the lysine 48 residue of the ubiquitin formerly added to the chain. The presence of four or more ubiquitins in a chain on a target protein is the indication for proteolysis via the 26S proteasome pathway.

2.2.2 E3 ligases

There are three families of E3 ligases: the homologous-to-the-carboxyl-terminus-of-E6-AP (HECT) family, the really-interesting-novel-gene (RING) family and the recently discovered U-box protein family (Hatakeyama and Nakayama, 2003). The RING-type E3 ligase can be categorized into two groups: they are either made up of a single subunit or multiple subunit complexes. Essentially, the various RING-type E3 ligase can be subcategorized into four other groups: the Skp1-Cul1-F-box protein (SCF), the anaphase-promoting complex/cyclosome (APC/C), the Cul2-ElonginB-

ElonginC complex and the single polypeptide RING-finger E3s (Hatakeyama and Nakayama, 2003).

The SCF ligase is made up of three invariable components: Skp1p, Cullin1p and Hrt1p/Rbx1p. F-box proteins (FBPs), which bind to Skp1p, are the variable components in the ligase. In the SCF complex, only Cullin1p that associates with the RING-finger protein Hrt1p, although there are six CUL genes identified in humans (Kipreos *et al.*, 1996). Skp1p acts as an adaptor, linking Hrt1p and Cullin1p to the various F-box proteins. One well-characterized human F-box protein is that of Skp2p, and thus the E3 ligase is termed SCF^{Skp2} where the superscript denotes the F-box protein. The structure of the SCF^{Skp2} quaternary complex is shown in Figure 5.

The N-terminal helical region of Cul1p is made up of three repeats of a novel structural domain called the cullin repeat. Essentially the cullin repeat is made up of five helices which are labeled A to E in Figure 4. In Cul1p, it is the first cullin repeat that comes into contact with Skp1p, whereas the C-terminus domain binds to Rbx1p/Hrt1p (Zheng *et al*., 2002). Interestingly, Cul1p can be modified by the conjugation of ubiquitin-like small molecule Nedd8, and neddylation of Cul1p has been observed to enhance the activities of SCF^{Skp2} and $SCF^{\beta\text{-}TrCP}$ (Ohh *et al*., 2002 and Amir *et al*., 2002).

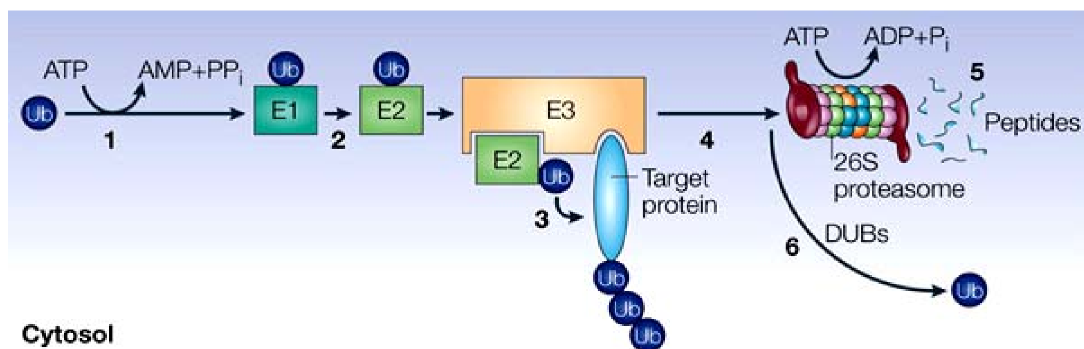

Figure 3: Ubiquitin conjugation and the ubiquitin–proteasome system. Reproduced with permissions from Nature Reviews Molecular Cell Biology Welchman *et al*., Ubiquitin and ubiquitin-like proteins as multifunctional signals, *Nature Rev. Mol. Cell Biol* 6 (8) 599-609, 2005; and Ciechanover, A. Proteolysis: from the lysosome to ubiquitin and the proteasome. *Nature Rev. Mol. Cell Biol*. 6, 79-87, 2005; copyright (2005) Macmillan Magazines Ltd

Ubiquitin is activated by the ubiquitin-activating enzyme (E1; step 1), and is subsequently transferred to an ubiquitin-conjugating enzyme (E2; step 2). In most cases, the E2 enzyme and the protein substrate both bind specifically to a particular ubiquitin-protein ligase (E3), and the activated ubiquitin moiety is then transferred to the protein substrate (step 3). The successive conjugation of ubiquitin moieties generates a polyubiquitin chain that functions as a signal to target the protein substrate to the 26S proteasome for degradation (step 4). The substrate is degraded to short peptides (step 5), and reusable ubiquitin is released by deubiquitylating enzymes (DUBs; step 6). P_i, inorganic phosphate; PP_i, pyrophosphate; Ub, ubiquitin.

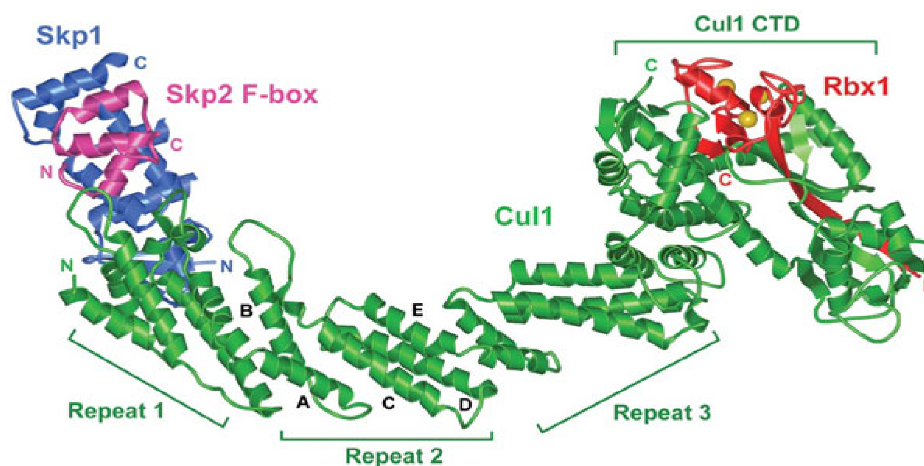

Figure 4: Overall structure of the Cul1-Rbx1/Hrt1-Skp1-F-box^{Skp2} quaternary complex. Reprinted by permission from Macmillan Publishers Ltd: Nature, Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex, Zheng *et al.* 416(6882): 703-9, copyright (2002).

Cul1, Rbx1/Hrt1, Skp1 and the F-box of Skp2 are colored in green, red, blue and magenta respectively. The five helices that make up the cullin-repeat motif are labeled for the second repeat. Figures were prepared with the programs MOLSCRIPT. GL_RENDER and POVRAY.

The SCF ligase is similar to another E3 ligase, the Cul2-ElonginBC complex (Lonergan *et al*., 1998 and Iwai *et al*., 1999). This family of E3 ligase consists of several proteins: Cul2p, RING protein Rbx1p/Hrt1p, ElonginB, ElonginC and a substrate recognition protein which contains a 10 amino acid degenerate sequence motif known as the BC-box. The substrate recognition protein acts as a linker between the substrate to be degraded and Cul2-ElonginBC; it is therefore functionally similar to the F-box protein in the SCF complex. This substrate recognition protein is the variable subunit in the E3 ligase, and examples of such proteins are the suppressor of cytokine signaling (SOCS)-box protein, ElonginA and the von Hippel-Lindau (VHL) tumor suppressor. The VHL protein was observed to be mutated in patients with the von Hippel-Lindau disease, and this results in the accumulation of the hypoxia-regulated transcription factor HIF1α. In turn, the increase in HIFα leads to transcription of hypoxia-inducible genes, thereby promoting tumor growth (Wykoff *et al*., 2001). A comparison of the SCF^{Skp2} and VHL-ElonginC-ElonginB complexes was done by Schulman *et al*. (2000). The ElonginC structure was noted to be similar to the N-terminal two-thirds of Skp1p, but it is missing the three helical domains which allows Skp1p to interact with Skp2p (Schulman *et al*., 2000). Thus Skp1p and ElonginC bind their substrate recognition partners differently. In addition, Yan *et al*. (2004) investigated the sequences in Skp1 and ElonginC that determine the binding to their cullin partners as Skp1p is known to bind to Cul1p whereas ElonginC binds to Cul2p. Short 11 amino acid structural elements in Skp1p and ElonginC were identified to be responsible for the selectivity in binding to different Cullins.

Interestingly, a novel interaction between ElonginC and mMed8p of the Mediator complex was discovered when a protein-protein interaction screen was carried out by Brower *et al*. (2002). A yeast-two hybrid screen using cDNA from human B-cell with ElonginC as bait revealed a mammalian BC-box protein that had homology to the *S. cerevisiae* Med8p. This BC-box sequence motif present in Med8p was the same as the domain present in the other substrate recognition proteins of ElonginBC-Cul2 E3 ligase like the VHL protein and ElonginA. In addition, Brower *et al.* (2002) also showed that the mammalian Med8p, Rbx1p/Hrt1p, Cul2p, ElonginB and ElonginC can reconstitute ubiquitin ligase activity, and that the mammalian Med8p can be copurified with other Mediator subunits.

2.2.3 S-Phase Kinase-Associated Protein 1A (Skp1)

There are several alternative titles to S-Phase Kinase-Associated Protein 1A (Skp1A), but presently, this is its most commonly used name. The other titles are the Cdk2/Cyclin A-Associated Protein, p19A, Organ of Corti Protein 2 (OCP2) and Transcription Elongation Factor B, 1-Like, (TCEB1L), and were related to how the SKP1A gene was discovered. In 1995, Chen *et al.* described the cloning of Ocp2 gene encoding OCP-II from a guinea pig organ-of-Corti cDNA library. Their results indicated that OCP2 had specialized roles in transcription in the inner ear. In the same year, there were two other reports of the same gene, but the link between them was not established then. Sowden *et al.* (1995) isolated a cDNA from human embryo libraries in a bid to find transcription factors that aid RNA polymerase II in the elongation phase. The cDNA was found termed Transcription Elongation Factor B, 1-Like (TCEB1L) as

it had significant homology to eukaryotic transcription factor Transcription Elongation Factor B (TCEB1). The latter is a subunit of the transcription factor SIII elongation complex. As for Zhang *et al.* (1995), it was the discovery of a protein of 19kDa associated with cyclinA-Cdk2 complex. As amino acid comparisons with databases did not yield any results, this 19kDa protein was termed S-Phase Kinase-Associated Protein 1 since it associates with the cyclinA-Cdk2 complex primarily during the S-phase of the cell cycle. In the end, it was the journal by Liang *et al.* (1997) that linked the three genes together in a bid to map the physical location of the gene. It should be noted that although Skp1p is not in direct interaction with cyclinA-Cdk2 complex; it requires another protein S-phase kinase associated protein 2 (Skp2) (Yam *et al*., 1999).

2.2.3.1 Functions of Skp1p in the SCF ligase complex

Bai *et al.* (1996) isolated SKP1 twice in separate screens in *S. cerevisiae*; it was originally isolated in a screen for suppressors of CDC34, an E2 ubiquitin ligase component necessary for the degradation of Sic1p. Subsequently, it was isolated in a two-hybrid assay to find interacting partners of Cyclin F; Cyclin F had previously been identified as a suppressor of cdc4-1 mutant (Bai *et al.*, 1994). The analysis of the cyclin F sequence revealed a novel motif termed the F-box domain to which Skp1p binds.

It was in 1997 that two groups showed that Skp1p was part of a complex which they term the Skp1p-Cullin/Cdc5-F-box (SCF^{Cdc4}) in which Cdc4p is the F-box protein (Skowyra *et al*., 1997 and Feldman *et al*., 1997). This E3 ubiquitin ligase was revealed to work with E1 and Cdc34p, an ubiquitin conjugating enzyme E2, to ubiquitylate S-phase Cdk inhibitor Sic1p (Feldman *et al*., 1997). Skowyra *et al.* (1997) also observed

that another F-box protein Grr1p was capable of reconstituting the SCF ligase with Skp1p and Cullin1p/Cdc53p. This E3 ligase targeted phosphorylated Cln1p rather than Sic1p.

The F-box protein (FBP) acts like a bridge linking the substrates to be degraded to the SCF ligase. Today, some 67 human F-box proteins have been identified (Jin *et al.,* 2004) and these proteins are categorized into three groups according to their substrate-binding domains they possess in addition to the F-box domain. These substrate-binding domains are found to be located at the carboxyl-terminal of the F-box proteins (Cenciarelli *et al*., 1999; Winston *et al*., 1999). One group of FBPs is termed FBWs whereby 'FB' is for F-box and 'W' for the WD-40 repeat domain. Those FBPs with leucine-rich repeats (LRRs) (Kobe and Kajava, 2001) are called FBLs, and the last group consist of proteins which do not fall into the above two categories, but contain a variety of protein motifs known as FBXs. In this third class of FBPs, protein-protein interaction domains like the proline-rich sequences, zinc-finger domain, and the helix-turn-helix sequences are present. However, the substrates for many of the F-box proteins are still not known.

One of the most well-known functions of the SCF complexes is the role they play in regulation of cell-cycle progression, specifically via the cyclins and their cyclin-dependent kinases (Cdks). The various F-box proteins Skp2p, β-Trcp1p, Cdc4p bring their substrates to the SCF ligase, resulting in their ubiquitylation and subsequently degradation. For instance, SCF^{Skp2} ubiquitylates phosphorylated p27, leading to the activation of Cdk1p and Cdk2p at the G1-S phase transition (Sheaff *et al.,* 1997; Montagnoli *et al*., 1999; Vlach *et al*., 1997). As for SCF^{Cdc4}, it targets cyclin E for

ubiquitylation; for cyclin E to be degraded, it must first be phosphorylated by Cdk2p and glycogen-synthase kinase 3β (Koepp *et al.*, 2001; Strohmaier *et al*., 2001).

2.2.3.2 Structure of Skp1p

Studies have been conducted to understand the interaction of Skp1 and the F-box protein. Schulman *et al*. (2000) published the crystal structure of Skp2p binding to Skp1p, and the structure is said to resemble a sickle where Skp1p and the F-box portion of Skp2p is the handle and the seven leucine rich repeats (LRR) of Skp2p looking like the blade (Figure 5). In Figure 5, the core interface referred to the region where the sequences of Skp1p and the F-box proteins are highly conserved in their respective families, whereas the variable interface is termed as such because the structural elements here are not conserved in their families. As mentioned earlier, Skp1p also interacts with Cul1p, through the N-terminal BTB/POZ domain fold. Biochemical studies by Ng *et al*. (1998) showed that dimeric form of Skp1p was observed, but the functions of these dimers in biological processes are unknown.

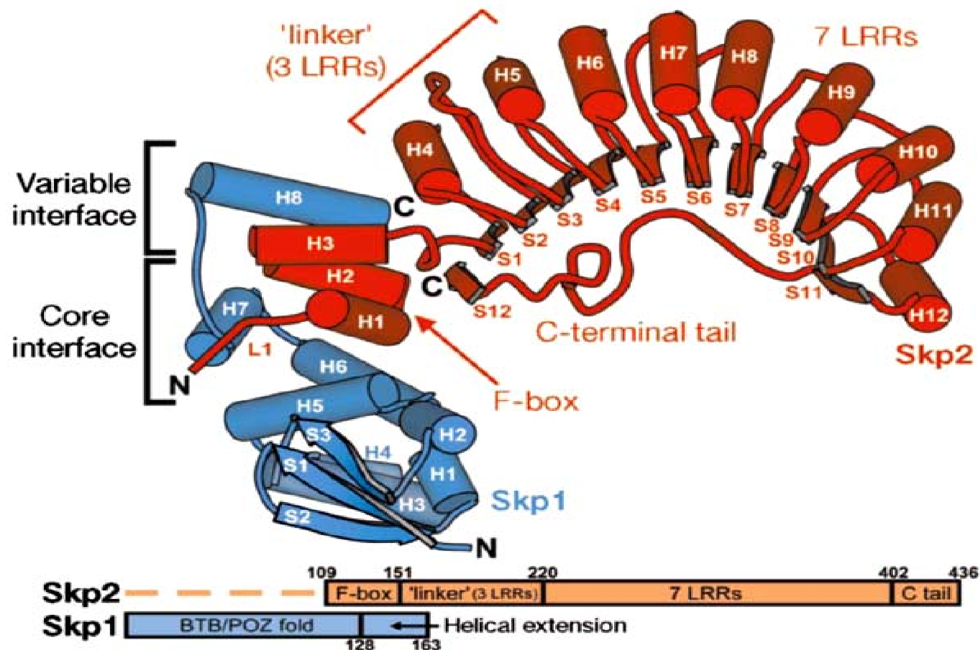

Figure 5: Structure of the Skp1p-Skp2p complex.
Reprinted by permission from Macmillan Publishers Ltd: Nature, Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex, Schulman *et al*. 408(6810), 381-6; copyright (2000).
Skp1p is shown in blue and Skp2p is shown in red. The boundaries of the BTB/POZ fold, the C-terminal helical extension of Skp1p and of the F-box, the three non-canonical LRRs, the seven canonical LRRs and the C-terminal tail of Skp2p are shown in the diagram below the structure. The 100-residue N-terminal Skp2p region missing from the crystallized protein is indicated (dashed line). The second LRR has a partially disordered loop instead of the helix characteristic of LRRs. (Taken from Schulman *et al*., 2000)

2.2.3.3 Skp1p: other functions other than in the SCF complex

From the various experiments done in yeast, it was clear that Skp1p had other functions either than its role in the SCF ligase. In 1996, Connelly and Hieter were searching for suppressors of kinetochore mutation *ctf13-30* in *S. cerevisiae*, and discovered a fourth subunit of the CBF3 kinetochore complex. These complexes are important for the attachment of chromosomes to the mitotic spindle, and are essential for high-fidelity segregation of chromosomes during cell division. They named the subunit suppressor of kinetochore protein 1, incidentally also abbreviated as SKP1. The

latter had high homology to the human homolog S-phase associated kinase (Skp1) found by Zhang *et al.* (1995) which was thought to play a role in cell cycle regulation (mentioned above). Thus Connelly and Hieter (1996) suggested that Skp1p is the link between kinetochore complex and cell cycle progression. It was later shown that ySkp1p interacts with Ctf13p, resulting in the phosphorylation of Ctf13p, which then activates the centromere-protein/transcription factor CBF3 kinetochore complex (Kaplan *et al.,* 1997). In a bid to better understand the yeast kinetochore complex, a screen was carried out to identify suppressors of *skp1-4* mutant (Kitagawa *et al.,* 1999). A novel Skp1 interacting gene was isolated from *S. cerevisiae*, and termed Suppressor of G2 allele of skp1 (SGT1). Subsequently, the interaction of ySgt1p and ySkp1p was found to be essential for the formation of the CBF3 kinetochore complex (Rodrigo-Brenni *et al*., 2004), and the interaction was regulated by Hsp90 chaperones (Lingelbach and Kaplan, 2004).

Skp1p has also been found to interact with another protein, F-box protein Rcy1p in *S. cerevisiae,* and the complex formed is important in endocytosis and recycling of plasma membrane sensitive attachment receptor protein (v-SNARE) (Galan *et al*., 2001). Although Rcy1p binds to Skp1p through its F-box domain, the interaction of Skp1p and Rcy1p does not form an SCF complex.

In a screen for novel interacting partners of ySkp1p and yCdc53p using mass spectrometry, two proteins were isolated and termed Rav1p and Rav2p (Seol *et al.,* 2001). It was found that these two proteins together with ySkp1p form a complex which the authors have called the ‘regulator of the H^+ ATPase of the vacuolar and endosomal membranes’ (RAVE). Both the Rav1p and Rav2p proteins did not have any F-box

sequence motif observed in the F-box proteins, indicating that Skp1p is able to bind to proteins without F-box motif. As the RAVE complex has no link to ubiquitin ligase activity, it is suggested that Skp1p is likely to have roles that are not related to ubiquitination.

The fact that proteins like Sgt1p, Rav1p and Rav2p do not have an F-box motif present in many of the proteins that interact with Skp1p indicates that Skp1p may have a wider role in different cellular processes either than its role in SCF E3 ligase ubiquitylation.

2.2.3.4 Evolutionary implications of Skp1

Studies have identified only one functional Skp1 protein in human and yeasts, but there are numerous Skp1 proteins identified in *Arabidopsis thalina, Caenorhabditis elegans, and Drosophila melanogastser* (Farras *et al*., 2001; Nayak *et al.,* 2002; Yamanaka *et al.,* 2002). Kong *et al.* (2004) has shown that in some plant and animal species which have multiple SKP1 homologs, the SKP1 gene has evolved at highly heterogeneous rates. Comparisons of the various protein sequences of Skp1p taken from Blast searches have been carried out, and it was found that no homolog of Skp1p exist in Eubacteria or Archaea.

2.2.4 Ubiquitin-proteasome system and transcription

The transcriptional regulation of genes and the ubiquitin-proteasome pathways are two major cellular processes which have been studied independently in the past. However, to get the whole picture of how genes are regulated, these two fields have now been linked together. Conventionally, the ubiquitylation of proteins has always

been a marker for its degradation by the proteasome complex, but recent investigations revealed there is more than meets the eye. The recent work on how ubiquitin-proteasome pathway and transcription are intertwined will be discussed below.

One clear example of how modification by ubiquitin affects cellular processes is the mono-ubiquitylation of histones like histones H2A in higher eukaryotes (Goldknopf *et al*., 1975), H2B in eukaryotes (West and Bonner, 1980), H3 in elongation spermatids of rat testes (Chen *et al*., 1998) and *Drosophila* H1 (Pham and Sauer, 2000). The modification of H2B with a single ubiquitin molecule has been recognized since fifteen years ago, but it was only recently that the function of this modification is understood (Zhang, 2003). Robzyk *et al.* (2000) first discovered that Rad6 is the enzyme responsible for the conjugation of a single ubiquitin moiety to histone H2B in *S. cerevisiae* at lysine 123. Subsequently, it was discovered that there was cross-talk between mono-ubiquitylation of H2B and the methylation status of lysine 4 and lysine 79 of histone H3 (Sun and Allis, 2002; Ng *et al*., 2002).

Interestingly, some complexes that are not related to the ubiquitin-proteasome system are now discovered to possess enzymatic activity that suggests otherwise. For instance, the Drosophila coactivator TATA-binding protein (TBP)-associated factor $TAF_{II}250$ was shown to be capable of mono-ubiquitylating linker histone H1 *in vitro* (Pham and Sauer, 2000). Mutations which affect the ability of TAF_{II} 250 to function as an ubiquitin-conjugating enzyme reduced the modified state of H1 and affected the transcription of genes by the maternal activator Dorsal. And there is the deubiquitylation of histone H2B by Ub-specific proteases (Ubps). For this, Henry *et al*. (2003) observed that Ubp8p, a component of the Spt-Ada-Gcn5-acetyltransferase

(SAGA) remodeling complex, is required for deubiquitylation of histone H2B. It is also intriguing to discover that the components of the proteasome complex, conventionally thought to function in the proteolysis of poly-ubiquitylated proteins, are involved in transcription. Specifically, mutations of the 19S regulatory particle Sug1 and Sug2 result in defects in transcription elongation as seen by the sensitivity of yeast cells to 6-azauracil (Ferdous *et al*., 2001). In fact, Sug1 had been isolated to interact with several transcription factors like Gal4, TBP and TFIIH (Swaffield *et al*., 1995; Melcher and Johnston, 1995; Weeda *et al*., 1997). One recent finding is that the 19S regulatory particle is able to regulate the SAGA co-activator via its ATPase activity, resulting in the stimulation of interactions between SAGA and transcription activators (Lee *et al.,* 2005). These findings suggest that a complex has a high probability of playing multiply roles due to the enzymatic activities of the various subunits it possesses.

There is one more piece of compelling evidence to strengthen the connection between ubiquitin-proteasome system and transcription, and that is the fact that there is an overlap between the transcriptional activation domains (TADs) and degradation signals known as degrons (Salghetti *et al*., 2000). It was shown that for unstable transcription factors like Myc and Gcn4p, the activation domain and destructive element possess overlapping sequences; this suggests that these transcription factors are degraded after the activation of transcription (Salghetti *et al*., 2001). Interestingly, the model suggested by Muratani *et al*. (2005) provides evidence that the activation of Gal4 target genes requires the poly-ubiquitylation of the transcriptional activator Gal4 and its proteolysis. In particular, the turnover of Gal4p affects the GAL1 mRNA at the

postinitiation step, therefore suggesting that this modification is required for the proper processing of transcripts.

Other than the ubiquitylation of transcription factors, the ubiquitylation of RNA polymerase II has also been reported; in cells with induced DNA-damaged, there is an increase in the ubiquitylated form of the RNA polymerase II (Ratner *et al*., 1998). This is known as the transcription coupled repair (TCR) where damage to actively transcribed DNA can be repaired in a process that involves the ubiquitylation and presumably the degradation of RNA polymerase II. Lee *et al*. (2002) showed a correlation between arrest of RNA polymerase II in transcription and ubiquitylation *in vitro*. In addition, Somesh *et al*. (2005) observed that transcriptional arrest which is not caused by DNA-damage can also result in the ubiquitylation of RNA polymerase II. Therefore, these results suggest a model that when the RNAPII undergoes transcriptional arrest, it becomes ubiquitylated and is presumed to be destroyed by the proteasome. In the event of DNA lesions, the degradation of polymerase II allows the repair machinery access to the damaged DNA. Therefore the expression of these genes is halted until the repair of the damaged DNA is completed. Similarly, RNA polymerase would be degraded if it is stalled during the elongation process as irreversibly stalled polymerase may compromise cell viability (Svejstrup, 2003). A proposed model of how the ubiquitin-proteasome pathway and transcription regulation are linked is shown in Figure 6 (Muratani and Tansey, 2003). In the model, an activator binds at the promoter region, and results in the recruitment of the ubiquitin ligase complexes. The latter would cause the ubiquitylation of proteins like the activator, histones and RNAPII. As a consequence, the proteasome complex is then recruited to the site, and destroys the

activator. In addition, the proteasome complex is also thought to convert the initiation form of RNAPII to the elongation form.

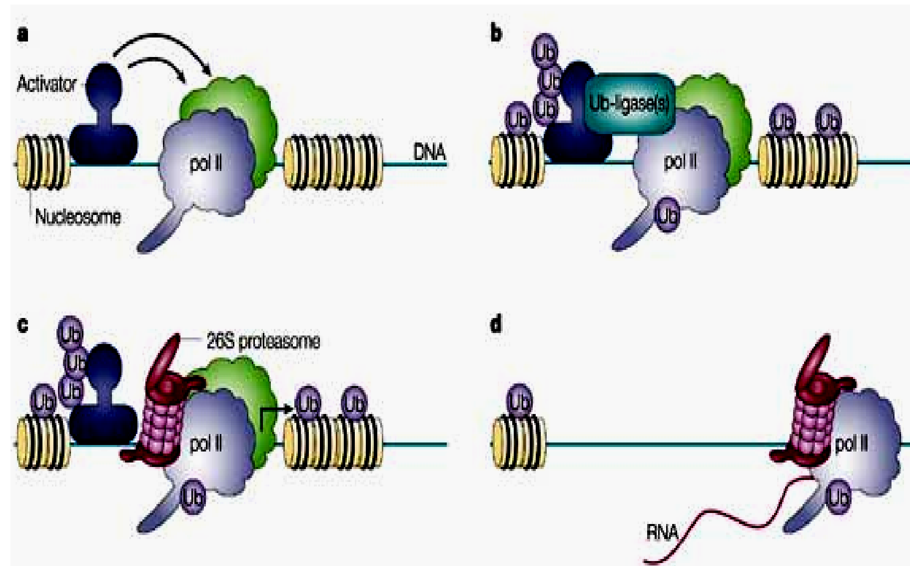

Figure 6: A unified model?
Reproduced with permission from Nature Reviews Molecular Cell Biology Muratani and Tansey, How the ubiquitin-proteasome system controls transcription, *Nature Rev. Mol. Cell Biol* 4(3); 192-201; copyright (2003) Macmillan Magazines Ltd.
In this model, the ubiquitin (Ub)–proteasome system regulates transcription at numerous levels. (a) Interactions of an activator with the general transcriptional machinery (green) functions to (b) recruit ubiquitin ligase(s) to the site of transcription and ubiquitylates many factors, including the activator, RNA polymerase II (pol II) and histones. (c) These ubiquitylation events in turn recruit the 26S proteasome, which (d) simultaneously destroys the activator and promotes elongation of transcription by pol II. Importantly, this proposed mechanism limits uncontrolled transcription in two ways — by destroying the activator at each cycle of promoter 'firing' and by ensuring that interactions between pol II and the proteasome are made in an activator- and promoter-dependent manner.

2.3 Protein-protein interaction systems

The yeast two-hybrid system developed by Fields and Song (1989) is probably the most widely used assay to study protein-protein interactions. This genetic assay is carried out in *Saccharomyces cerevisiae*, and its principle is based on the fact that a transcription factor can be divided into two domains that are functionally distinct: the DNA binding domain (DBD) and the transcription activation domain (AD) (Brent and

Ptashne, 1985; Keegan *et al*., 1986). If these two domains are physically separated and do not interact, transcription will not occur on responsive genes. Activation of transcription would only occur when the DBD domain and AD domains are brought together. Thus based on the these observations, Fields and Song (1989) realized that they could have a pair of interacting proteins each fused to either domain of the yeast Gal4 transcription factor, and so the assay was established. The original yeast two-hybrid system is based on transcriptional readout of a single reporter gene, but in the last sixteen years many other variants of the original system has surfaced. Yeast strains carry more than one reporter gene to decrease the chances of having false-positives and there are also systems using the DNA binding domain of bacterial repressor protein LexA with the activation domain from *Escherichia coli* (Causier, 2004). However, there are several disadvantages in using such systems. Firstly, protein interactions in the yeast two-hybrid systems must occur in the nucleus, thus the analysis of hydrophobic proteins like trans-membrane proteins would be futile. As such, false negative results may arise. Secondly, the assay excludes transcription activators as these proteins may interact with the transcription machinery and cause the false positives in the absence of true protein-protein interaction; transcriptional repressors are not suitable, too.

Other methods to test for interacting proteins *in vivo* other than yeast two-hybrid systems have been published. Two examples are the son of sevenless (SOS) recruitment system (SRS) (Aronheim *et al*., 1994) and Ras recruitment system (RRS) (Broder *et al*., 1998). Unlike the yeast two-hybrid assays that depend on transcription readouts, these protein recruitment methods are monitored by cell viability in a temperature sensitive yeast strain. Therefore, these systems can be employed to study

transcriptional factors. The main disadvantage of these assays is that the use is restricted to either nuclear or cytoplasmic proteins, and membrane proteins cannot be analyzed (Aronheim, 2000). On the other hand, these methods enable us to map domains of proteins which are responsible for the translocation of the proteins to the plasma membrane. Recently, the development of the reverse RRS (Hubsman *et al.,* 2001) now allows the use of membrane proteins as bait. This assay can be used for characterization of known interactions as well as isolation of new interacting partners in a library screen approach. The RNA polymerase III based two-hybrid system is another assay that can be used in the study of transcription factors (Marsolier *et al*., 1997). This system is based on the fact that different transcription factors are involved in the regulation of RNA polymerase II and RNA polymerase III.

2.3.1 Mammalian protein-protein interaction systems

Although there are several systems available to screen mammalian proteins in *S. cerevisiae*, it would still be best to validate the protein-protein interactions in mammalian cells. This is because the modification system in yeast differs from mammalian cells, and in higher eukaryotes, the conditions necessary for folding, processing and activation of mammalian proteins are already present. Several methods to detect protein-protein interactions in mammalian cells have been established and are discussed below.

The mammalian protein-protein interaction trap (MAPPIT) based on cytokine receptor developed by Eyckerman *et al.* (2001) is an increasingly popular screening method in mammalian cells. This cytokine receptor-based two-hybrid method is based

on JAK and signal transducers and activators of transcription (STAT) signaling pathway. The key advantage of this system is the detection of modification dependent protein-protein interactions; this is because the read-out is ligand-dependent. Another novel strategy of examining protein-protein interaction in mammalian cells is based on receptor engagement, and therefore termed selection of protein interactions by receptor engagement (SPIRE) (Ellmark *et al.*, 2004). Here, the proteins are expressed on the surface of mammalian cells, which therefore allows the detection of proteins that are expressed on the extracellular side. One of the latest assays to probe for interacting partners in mammalian cells is the dual-light reporter system (Nasim and Trembath, 2005). One key feature of this system is that it does not depend on a single reporter function; this is advantageous as it does not rely on transfection efficiencies. Instead the method is comprised of two independent gene expression units under the same promoter on a single plasmid. The upstream reporter is transcribed once the plasmid is introduced into mammalian cells, whereas the downstream reporter would only be expressed as a result of an interaction. Subsequently, a ratio of the two reporter gene can be obtained for semi-quantitation purposes.

2.3.2 Other new technologies to study protein-protein interactions

Currently, the key technologies used to identify proteins and their interacting partners, and to characterize the interactions include the 2-D electrophoresis, mass spectrometry and multidimensional protein identification technology (MudPIT) (Washburn *et al.*, 2001). There are also the high throughput technologies available to study protein-protein interactions on a large scale. For instance, protein array chips are

gaining popularity as an *in vitro* screening method. Other *in vivo* methods include fluorescence resonance-energy transfer (FRET) and high through-put yeast two-hybrid based on mating of yeast (Wallrabe and Periasamy, 2005).

2.3.3 The split-ubiquitin system

The split-ubiquitin system is the most commonly used alternative to the standard yeast two-hybrid assay (Auerbach *et al*., 2002), and it was originally developed by Johnsson and Varshavsky (1994).

2.3.3.1 Principles of the split-ubiquitin system

The original assay is based on conditional proteolysis that occurs upon the reassociation of the N- and C- terminal halves of ubiquitin designated N_{ub} and C_{ub} respectively. Each half of ubiquitin is fused to either protein of interest. Should the two proteins interact, the two halves of ubiquitin would be brought together in close proximity. This reconstituted native-like ubiquitin is recognized by ubiquitin specific proteases (UBPs), and a reporter attached to C_{ub} is then released. As a result, the reduction in size of the reporter protein can easily be detected through Western blot (Dünnwald *et al*., 1999). Because UBPs are present in the cytosol as well as in the nucleus (Varshavsky, 1997; Byrd *et al*., 1998), this assay can be used to probe protein-protein interactions that do not occur in the nucleus. This overcomes a limitation of the standard yeast two-hybrid assays in that protein-protein interactions are required to occur in the nucleus. Thus the split-ubiquitin assay has been used to investigate membrane proteins in their native environment (Dünnwald *et al*., 1999). Another

difference between conventional yeast two-hybrid system and the split-ubiquitin assay is that the latter does not depend on transcriptional readout. This enables the application of split-ubiquitin system in the analysis of strong transcription factors as seen in the work by Wellhausen and Lehming (1999).

To decrease the chances of having spontaneous interactions between N_{ub} and C_{ub} without any actual interaction of the two proteins, some N_{ub} mutants were constructed (Johnsson and Varshavsky, 1994). This results in a more sensitive assay, as it allows us to adjust for the background level. Wild type N_{ub} carries an isoleucine in position 13, and the two mutations were created such that N_{ub} now carries an alanine ($N_{ub}A$) or glycine ($N_{ub}G$) at that position. The strength of interaction with C_{ub} as compared to wild type N_{ub} is reduced in $N_{ub}A$, and for $N_{ub}G$, it is further decreased. This allows us to have some quantitative analysis of the strength of protein-protein interactions.

This assay enables the detection of interactions between two proteins, but has a disadvantage in that it is not possible for the selection of new interacting partners from random fusion library. Two variants of the original assay have been reported to date: one is based on conditional compartmentalization (Stagljar *et al*., 1998), while the basis of the other strategy is conditional protein degradation (Wittke *et al*., 1999).

2.3.3.2 The conditional compartmentalization design

The basis of this design is that a transcriptional activator can only exert its function if it is present in the nucleus. In the report by Stagljar *et al*. (1998), Wbp1p, a transmembrane protein anchored at the endoplasmic reticulum (ER) was fused to C_{ub} and the artificial activator LexA-VP-16. The latter is able to bind to LexA binding sites

(Vojtek *et al*., 1993) upstream of reporter genes like *HIS3* and *lacZ*, thereby activating their transcription. This tripartite fusion of Wbp1-C_{ub}-LexA-VP16 was retained at the membrane and is unable to enter the nucleus. Thus the yeast was not able to grow on plates that lack histidine, and when plated on X-gal plates, white colonies were observed. Ost1p was the other protein fused to N_{ub}. Both Wbp1p and Ost1p are essential components of the yeast oligosaccharyltransferase complex, and the co-expression of these two proteins with their fusion partners resulted in their interaction. Consequently, the two halves of ubiquitin reassembled into a native-like ubiquitin, and the Ubps cleaved off the LexA-VP1 fusion. This activator then entered the nucleus and activated the transcription of the two reporter genes; the yeast was now able to grow on plates lacking histidine, and blue colonies were observed upon plating onto X-gal plates.

The advantage of this assay is that the same yeast strain used in conventional yeast two-hybrid assays is applicable here (Vojtek *et al*., 1993). However, this study is limited as only proteins that do not localize to the nucleus can be tested.

2.3.3.3 The conditional degradation design

This strategy was first described in *S. cerevisiae* by Wittke *et al*. (1999), and subsequently adapted for mammalian cells (Rojo-Niersbach *et al*., 2000).

The main principle of this system is the N-end rule, which states that the N-terminal amino acid mediates the *in vivo* half-life of a protein (Varshavsky, 1996). This pathway is present in organisms like *Escherichia coli, S. cerevisiae,* and mammalian cells. In eukaryotes, the N-end rule is part of the ubiquitin system, and in addition to a destabilizing N-terminal residue, an internal lysine is required (Varshavsky, 1997). The

destabilizing amino acid residues in *S. cerevisiae* fall into two groups: basic residues like arginine, lysine and histidine, or bulky hydrophobic residues, like leucine, phenylalanine, tyrosine, tryptophan and isoleucine. There are also stabilizing N-terminal residues like methionine, alanine, glycine, serine, threonine, cysteine, valine and proline (Varshavsky *et al*., 2000). Ubr1p, a RING finger-containing E3 ubiquitin ligase, recognizes the N-terminal residues; this enzyme is responsible for the addition of a multi-ubiquitin chain to the internal lysine residue of the substrate (Dohmen *et al*., 1991). As a result, the ubiquitylated substrate is degraded by 26S proteasome, an ATP-dependent multisubunit protease. Arginine is the most destabilizing residue as shown by Varshavsky (1995), and thus this property is exploited in subsequent split-ubiquitin systems where the first residues of reporter proteins have been substituted with an arginine.

For the selection system in yeast (Wittke *et al*., 1999), the first amino acid in reporter protein orotidine-5'-phosphate decarboxylase (Ura3) had been replaced by arginine (RUra3). One protein was fused to N_{ub}, while a second protein was fused to C_{ub}-RUra3. Should the two proteins interact inside the cell, a native-like ubiquitin would be formed, and the Ubps cleave off the RUra3 moiety. As a result, the reporter protein was rapidly degraded and the phenotype of the yeast becomes uracil-deficient. Protein-protein interactions can be selected for with the help of 5-fluoroorotic acid (5-FOA), a drug counterselecting Ura3 enzymatic activity. Figure 7 shows the events that occur in a split-ubiquitin screen using Gal4 as bait (Laser *et al*., 2000).

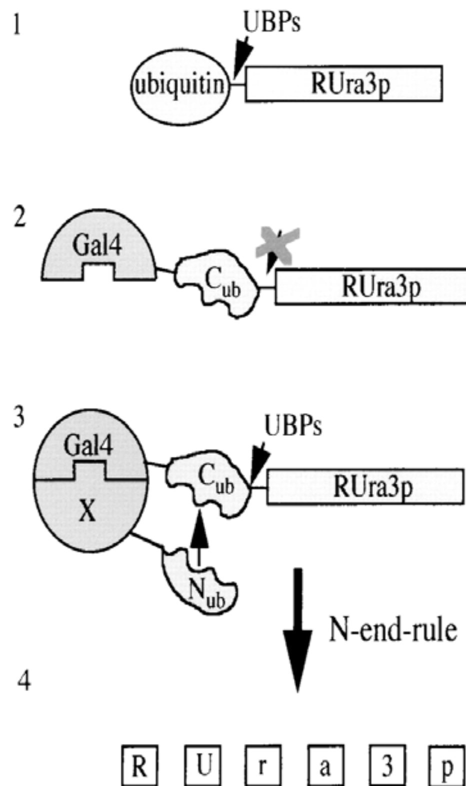

Figure 7: The split-ubiquitin system in yeast based on the conditional degradation design.

Reproduced from PNAS, A new screen for protein interactions reveals that the Saccharomyces cerevisiae high mobility group proteins Nhp6A/B are involved in the regulation of the GAL1 promoter, Laser *et al.*, 2000, 97(25), 13732-13737; Copyright (2000) National Academy of Sciences, U.S.A.

Ubiquitin, fused to the N terminus of the Ura3 displaying an arginine as its first amino acid (RUra3), is recognized by the UBPs (line 1). A fusion protein containing Gal4, C_{ub} and the reporter RUra3 is not cleaved by the Ubps (line 2). The fusion is enzymatically active and the yeast cells are phenotypically uracil prototrophic and sensitive to 5-FOA. The co-expression of N_{ub} fused to a protein X together with Gal4-C_{ub}-RUra3 leads to an increase in the local concentrations of N_{ub} and C_{ub} if Gal4 and X interact inside the cell. A native-like ubiquitin is formed, and the Gal4-C_{ub}-RUra3 fusion is cleaved. The enzymes of the N-end rule degrade the free RUra3 reporter rapidly. Therefore the protein interaction between Gal4 and X can be detected by the absence of growth on plates lacking uracil and by the growth on plates containing 5-FOA.

This set-up has been used to screen a genomic library consisting of *S. cerevisiae* fragments fused to N_{ub} as the prey and the transcriptional repressor Tup1p and the transcriptional activator Gal4p as baits (Laser *et al*., 2000; Pätzold and Lehming, 2001; Kerkmann and Lehming, 2001). In the screen performed with Gal4p as bait, four new binding partners were discovered (Laser *et al*., 2000), and in the screen with Tup1p as bait yielded 12 novel binding partners for this transcriptional repressor (Kerkmann and Lehming, 2001). However, some artifacts emerged in the screening process, and some of these proteins interacted with the C_{ub} part of the bait. The artifacts include Gog5p and Ymd8p which are small molecule transporters that cause FOA resistance when

overexpressed, ubiquitin hydrolase Mum2p and ubiquitin conjugating enzyme Ubc1p. Furthermore the protein-protein interactions detected by the split-ubiquitin assay may not be due to direct interactions, but due to close proximity of the two proteins. Thus the isolated proteins have to be tested *in vitro* to determine if their interactions are direct ones. All proteins pair tested were found to be direct interactions except for Tup1p and Cdc73p. Further work carried out by Kerkmann and Lehming (2001) revealed that Cdc73p and Tup1p regulate a common set of genes. Thus the screen had identified the close proximity of the two proteins as they co-occupy the promoter even though these two protein were not directly interacting. It was also shown that the screening of the genomic library could not detect all possible protein interactions. Gromöller and Lehming (2000) showed that indeed Tup1p interacts with Srb7p, a essential subunit of the holoenzyme for transcription. Because the screening of the library had been carried out such that the N-terminus of Srb7p was fused to N_{ub} and therefore creating N_{ub}-Srb7 fusion, the interaction between Srb7p and Tup1p was not detected previously as the free N-terminus of Srb7p was required for the interaction.

A third class of reporter gene has been developed using the green fluorescent protein (GFP) fused to C_{ub} (C_{ub}-RGFP) (Laser *et al*., 2000). Theoretically, this system does not limit the study of protein-protein interactions to either yeast or mammalian cells, and is generally applicable to all eukaryotic cells. Gromöller and Lehming (2000) have applied this variant of the split-ubiquitin system to test the interactions between Tup1p and its known potential targets. *S. cerevisiae* cells expressing Srb7-C_{ub}-RGFP show strong nuclear green fluorescence. When N_{ub}-Tup1p was co-expressed with Srb7-C_{ub}-RGFP, the nuclear localized GFP signal was destroyed. This indicated that the

interaction between Tup1p and Srb7 caused the local concentrations of C_{ub} and N_{ub} to increase, and subsequently native-like ubiquitin were formed. Cleavage by Ubps leads to the degradation of the GFP reporter by the N-end rule enzymes.

2.3.3.4 Modified split-ubiquitin systems in mammalian cells

As for the application of the split-ubiquitin system in mammalian cells, a different reporter gene was used. Rojo-Niersbach *et al*. (2000) have shown the use of an RGpt2 reporter protein in selection of protein interactions in the human fibroblast cell line HT1080HPRT$^-$ (Pellegrini *et al*., 1989). This cell line lacks the enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT), and is therefore sensitive to medium containing hypoxanthine/aminopterin/thymine (HAT), but is resistant in medium containing 6-thioguanine (6TG). The guanine phosphoryltransferase 2 (gpt2) gene of *E. coli* is able to complement the deficiency. This system was used to investigate the interaction between human TATA-binding protein 1 (hTBP1) and the C-terminal domain of human nuclear factor κB (NF-κB) termed p65C, and interaction between *Homo sapiens* nuclear autoantigen (hSP100B) and human heterochromatin protein 1α (hHP1α) in living cells. These two interactions had been previously characterized *in vitro* (Kerr *et al*., 1993; Lehming *et al*., 1998; Seeler, *et al*., 1998). Figure 8 shows a schematic representation of system set-up. The HT1080HPRT$^-$ cell line expressing the tripartite fusion of p65C-C_{ub}-Rgpt2 alone should display HAT resistance and 6TG sensitivity (Figure 8a). N_{ub}-hTBP1p was then co-expressed in the cells. Should p65C and hTBP1p interact *in vivo*, the two proteins would be brought in close proximities (Figure 8b). This resulted in the reconstitution of the ubiquitin, and

Ubps would cleave the p65C-C_{ub}-RGpt2 fusion (Figure 8c). Thus, the RGpt2 reporter protein was rapidly degraded by enzymes of the N-end rule (Figure 8d). This lead to a change in phenotype of the cell line to HAT-sensitive and 6TG resistant.

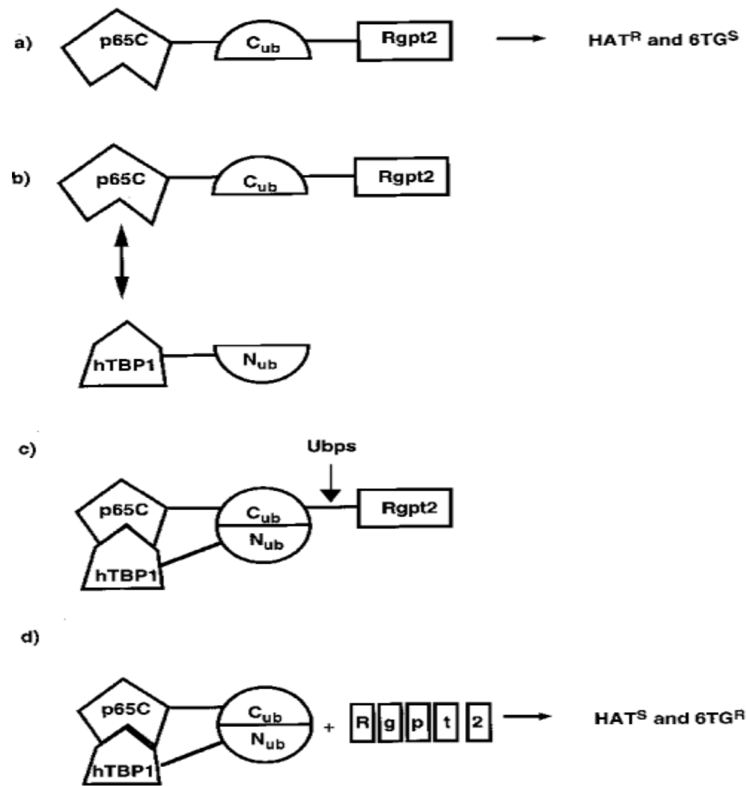

Figure 8: Schematic illustration of the mammalian split-ubiquitin system. Reproduced with permission from Rojo-Niersbach *et al.* (2000), A new method for the selection of protein interactions in mammalian cells, 348, 585-590, © the Biochemical Society.

(a) An HT1080HPRT$^-$ cell line expressing p65C-C_{ub}-Rgpt2 is HAT-resistant (HAT^R) and 6TG sensitive ($6TG^S$). (b and c) Should N_{ub}-hTBP1 be co-expressed, hTBP1 and p65C bind to each other inside living cell. As a consequence, native-like ubiquitin is reconstituted and the p65C-C_{ub}-Rgpt2 fusion protein is cleaved behind C_{ub}. (d) The cleavage product Rgpt2 is rapidly degraded by enzymes of the N-end rule, thus resulting in HAT-sensitivity and 6TG-resistance.

2.3.3.5 Recent applications of the split-ubiquitin systems

The modified split-ubiquitin system based in yeast has been widely used to study membrane proteins involved in signaling and transporters in yeast, plants and

mammals, and to investigate interacting partners of membrane proteins with enzymatic functions like oligosaccharyl transferase in yeast (Pasch *et al.*, 2005; Yan *et al.*, 2005; Pandey and Assmann, 2004; Schulze *et al.*, 2003). This modified split-ubiquitin system has also been used for studying of viral membrane proteins like the Hepatitis B surface antigen (HBsAg) in understanding viral morphogenesis (Toh *et al.*, 2005). In addition, the split-ubiquitin system has also been used to verify interactions between two membrane proteins. Wang *et al.* (2004) was reportedly the first group to use the yeast split-ubiquitin system based on the compartmentalization approach to screen a cDNA library. They used the endoplasmic reticulum membrane protein BAP31p as bait to screen a cDNA library for interaction proteins, and a novel human member of the protein tyrosine phosphatase-like B (PTPLB) family was isolated.

In addition, the large-scaled analysis of the integral membrane proteins of *Saccharomyces cerevisiae* was recently carried out by Miller *et al.* (2005) using the modified split-ubiquitin system. A total of 705 membrane proteins were short-listed for protein-protein interaction analysis, but only 365 proteins were found to be competent for use in the screening. Of a total of 270 screens conducted, 1, 985 interactions were observed. The group then went on further to use a learning algorithm, support vector machine (SVM) to classify the interactions to different levels. This study was successful in isolating novel previously undiscovered interacting candidates. As such, the coupling of computational tools together with experimental data in large-scale protein-protein interaction studies is useful in weeding out the false-positive and false-negative interactors. However, like for most protein-protein interaction screens, one must perform other experimental strategies to confirm the results.

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Molecular Cloning

3.1.1 Construction of primers

The genomic sequence of each gene was obtained from GenBank (National Institute of Health); for hSKP1A, the Accession number was NM_179679. PCR primers were constructed such that they were at least 20 base pairs long, and had an annealing temperature of greater than 50ºC. Different 3' antisense primers for the construction of N_{ub} and C_{ub} fusions were made. For the C_{ub} 3' antisense construct, the stop codon was removed, while for the N_{ub} construct, the stop codon remained unchanged. The 5' sense primer was used with either one of the 3' antisense primers for any one PCR reaction. Sequences of restriction enzymes were included in the primers; this allows for the ease of inserting this PCR fragment into the vector. The sequences of the PCR primers are listed in Table 1.

ORFs	Primer name	PCR primer sequences	Length of transcript (bp)
hSKP1A	5' BamMun 3' XhoIgo 3' SalIstop	5'- gccggatcccaattgatgccttcaattaagttgca-3' 5'- gccgccctcgagttctcttcacaccactggt-3' 5'- gccgccgtcgactcacttctcttcacaccact-3'	492
hSRB7	5' MunI 3' XhoIgo	5'-gcccaattgaaaatggcggatcggctcacgca-3' 5'-gccgcctcgaggcgtttgagtctggaagagactggc-3'	435
hSGT1	5'BamH I 3' NotI	5'-gccgccggatccatggcggcggctgcagcagg-3' 5'gcgccgccgccgcttagtactttttccattccata-3'	1171

Table 1: List of primers for PCR of full-length hSKP1, hSRB7 and hSGT1.

3.1.2 Polymerase Chain reaction (PCR)

Amplification of the DNA was carried out using Expand High Fidelity PCR system (Boehringer Mannheim). The components of each tube were prepared as

indicated in Table 2. A cDNA library derived from human B cell was used as a PCR template for the synthesis of all open reading frames (ORFs). The samples were then amplified for 35 cycles. First, the cycler was preheated at 94ºC for 2 minutes before amplification began. Then the program for each cycle started at 94ºC for 1 minute, followed by 50ºC for 1 minute, and 72ºC for 2 minutes. The samples were then cooled at 4ºC before electrophoresis on an agarose gel.

Reagent	**Volume (µl)/ 1 sample**	**Final Concentration**
10 X PCR buffer with $MgCl_2$	5	1X
dNTP	1	0.2mM
Primer 1 – 5' sense	0.5	50µM
Primer 2 – 3' antisense	0.5	50µM
Template DNA	0.1	2 µg/µl
Sterile water	42.4	--
High fidelity polymerase	0.5	1 unit
Total volume	50	--

Table 2: Components of each PCR reaction sample.

3.1.3 Agarose gel electrophoresis

A 1% agarose gel was cast using 100ml of 1 x Tris Borate EDTA (TBE) buffer and 1g of agarose (GibcoBRL, Life Technologies). 3µl of ethidium bromide (Bio-Rad Laboratories) was added to the gel to allow the direct visualization of the DNA in the gel. 10µl of PCR samples were loaded with 2µl of 6 times loading dye, and pGEM DNA marker (Promega Corporation, USA) was used. The gel was electrophorized at a constant voltage of 100 volts for 45 minutes, and then viewed under UV (GeneGenius, Syngene).

3.1.4 DNA purification

Only PCR fragments that are of the expected size were subsequently purified. Restriction digestion mixes were also subjected to DNA purification after incubation to remove the enzymes. This process was carried out using High Pure PCR Product purification kit (Boehringer Mannheim), and according to manufacturer's protocol. The purified DNA was resuspended in appropriate amounts of sterile water.

3.1.5 Restriction digestion of plasmids and inserts

The plasmids for mammalian work used have been described previously (Rojo-Niersbach *et al*., 2000). The C_{ub} vector had a *Xho*I/*Apa*I-cut fragment containing the C-terminus of ubiquitin fused to guanine phosphoryltransferase 2 (Gpt2), modified to begin with an arginine residue, placed in pcDNA3 + zeocin plasmids (Invitrogen). As for the N_{ub} fusion vector, a *Hind*III/*Eco*RI-restricted PCR fragment containing N_{ub} had been inserted into pcDNA3 (Invitrogen). The PCR fragments of Srb7 was cleaved with *Mun*I/*Xho*I, and inserted into the pcDNA3-C_{ub}-RGpt2 vector that was cut with *Eco*RI and *Xho*I. The PCR fragment of Skp1 was cleaved with *Mun*I/*Sal*I, and ligated with pcDNA3-N_{ub}I that was cut with *Eco*RI and *Xho*I. The reaction was carried out overnight at 37ºC in a total volume of 100µl, and consisted of 20µl of the purified PCR fragments, and 1µl of each enzyme used for the restriction. The expression plasmids for *S. cerevisiae* have been described previously in Laser *et al*. (2000). The plasmids are the single-copy P_{CUP1}- C_{ub}-RUra314 fusion vector and the single-copy pACNX-N_{ub}IBC. Cloning of hSkp1 into the C_{ub}-RUra3 vectors was between the restriction sites *Bam*HI

and *Sal*I site. As for the N_{ub} fusion vectors, they were cut with *Eco*RI and *Not*I enzymes overnight and purified as described above.

3.1.6 Ligation and transformation into *Escherichia coli* cells

Ligation was carried out by mixing 1µl of the cut vector, 3µl of cut fragment and 0.5µl of the T4 ligase (Boehringer Mannhein, Germany) in a 1.5ml microtube. The reaction was topped up with 1µl of 10X ligation buffer (Boehringer Mannhein, Germany), and 4.5µl of sterile water, bringing the total volume to 10µl. This mix was placed overnight at 4°C.

Transformations of the vectors into competent DH5α *E. coli* cells were carried out to amplify the plasmid. To 50µl of vector mix, 50µl of DH5α was added and the mixture was incubated on ice for 20 minutes. Following this, the mixture was placed in the water bath at 42°C for 90 seconds. 400µl of Luria Bertani (LB) was added to the mixture and the tube was placed at 37°C for 1 hour. All 250µl were streaked onto LB agar with ampicillin (50mg/ml), and placed overnight at 37°C.

3.1.7 Screening for positive transformants

The preliminary screening for transformants was performed by plasmid minipreps using alkaline lysis method (Ausubel *et al*., 2006), and followed by restriction digestion analysis.

5µl of the isolated DNA was then taken for a restriction digest to check if there was the correct insert. 0.25µl of each restriction enzyme was used, and the reaction was topped up to a final volume of 25µl with sterile distilled water and restriction buffer.

The restriction digestion reaction was placed at 37°C for two hours before the samples underwent electrophoresis on a 1% agarose gel as stated in Section 3.1.3. The plasmid that generated DNA fragment with the expected size after digestion was stored at -20ºC for retransformation in large scale preparation of plasmid DNA.

3.1.8 Large scale preparation of plasmid DNA

Retransformation of positive transformants was carried prior to the large scale preparation of plasmid DNA. 0.5µl of the plasmid from earlier analysis was incubated with 10µl of competent DH5α cells on ice for 20 minutes. This mixture was then placed in a 42ºC waterbath for 90 seconds. 40µl of LB solution was added to the mixture that was next placed at 37°C for 1 hour. 10µl of the solution was then plated onto LB agar with 50µg/ml ampicillin. The plasmids were then subjected to purification using the maxi kit (Qiagen), or by alkaline lysis and subjected to cesium chloride/ethidium bromide purification (Ausubel *et al*., 2006).

3.1.9 Sequencing of samples

Subsequently, DNA sequencing was carried out. For the DNA sequencing of cDNA3-Srb7-C_{ub}-RGpt2, the forward primer that hybridizes to the T7 promoter was 5'-taatacgactcactataggg-3', and the reverse primer sequence that hybridized in the C_{ub} region (C_{ub}50) was 5'-cagacagcgttctacgtct-3'. As for pcDNA3-N_{ub}1-Skp1, the forward primer (N_{ub}100) was 5'-cgttaagtcgaaaattcaag-3', and the reverse primer (TcDNA3) was 5'-ggggaggggcaaacacaga-3'. Only one primer was used in each reaction, and cycle sequencing followed by the purification of the extension products was carried out as

followed. To 250ng of template DNA, 1.6ρmol of primer and 2µl of Terminator Ready Reaction Mix were added. The reaction was topped up with deionized water to 10µl. The following cycle parameters were used: 96ºC for 30 seconds, 50ºC for 15 seconds, and 60ºC for 4 minutes. Upon completion of the reaction, the contents were each transferred into a 1.5ml microtube that contains 80µl of ethanol/sodium acetate solution (consisting of 3.0µl 3M sodium acetate, pH4.6, 62.5µl of nondenatured 95% ethanol and 14.5µl of deionized water). The microtubes were then vortexed briefly, and left at room temperature for 15 minutes to precipitate the extension products. Next, the tubes were centrifuged at 13,000 rpm for 10 minutes, and the supernatant was carefully aspirated with a pipette and discarded. 500µl of 75% ethanol to rinse the pellet, and the microtube was centrifuged at 13,000 rpm for 5 minutes. The supernatant was then discarded, and the pellet was dried in a speedvac concentrator for 10 minutes. Subsequently, the dried samples were given to Department of Microbiology (National University of Singapore) for DNA sequencing.

3.1.10 Cloning of small-interfering RNA (siRNA) constructs

The pSuper RNAi system (OligoEngine) was used to direct the intracellular synthesis of siRNA-like transcripts in a mammalian expression vector. The plasmid was a kind gift from Dr Ng Huck Hui (Genome Institute of Singapore). A unique 19-nucleotide sequence was derived from the hSkp1 target gene (Accession number NM_179679) with the help of Whitehead Institute's siRNA prediction tool (http://jura.wi.mit.edu/bioc/siRNAext/). The lists of 19-nucleotide sequences were then

subjected to further analysis based on criterions stated in these two articles: Hsieh *et al*. (2004) and Reynolds *et al*. (2004).

The forward and reverse oligo were generated such that they had a *Bgl*II site at the 5' end, while the 3'end contained the T5 sequence and any corresponding *Sal*I site. The target sequence is as shown in Table 3.

siRNA	Sequence	Target
hSkp1	5'-gcactgctctgtttataat -3'	681-703 in the 3' untranslated region

Table 3: The 19-nucleotide target sequence of hSkp1.
This sequence was targeted for suppression using the pSUPER RNAi system.

The full length 60bp forward oligo sequence was 5'-GATCCCCGCACTGCTCTGTTTATAATTTCAAGAGAATTATAAACAGAGCAGTGCTTTTTG-3', and the full length 60bp reverse oligo sequence was 5'-TCGACAAAAAGCACTGCTCTGTTTATAATTCTCTTGAAATTATAAACAGAGCAGTGCGGG-3'. 1μg of the forward and reverse oligo (1st Base) each was annealed using annealing buffer (100mM potassium acetate, 30mM HEPES and 2mM magnesium acetate) and incubated at 95°C for 4 minutes. Subsequently, the oligos were incubated at 70°C for 10 minutes and then at room temperature for an hour. Ligation was carried out using pSuper that had been digested with *Bgl*II and *Sal*I, and transformed into *E. coli* DH5α strain and selected on LB plates containing ampicillin. Plasmids were subjected to alkaline lysis and gel electrophoresis to verify the presence of the insert. DNA sequencing was carried out using these two primers to verify that cloning was successful: Forward primer: 5'-tcgctatgtgttctgggaaa-3' and reverse primer: 5'-gctatgaccatgattacgcc-3' hybridizing to the pSuper plasmid were used for the DNA sequencing. It was ensured that the entire 60bp of insert was present, and there were no

mutations present. As the plasmid targeted region 681-703, it was named pSuper-hSkp1-681. Transfection into 10^6 HeLa cells was carried out using Lipofectamine™ 2000 (Invitrogen). Subsequently, Western Blot was carried out using an antibody against hSkp1 (Abcam, ab10546).

3.1.11 Isolation of total RNA

Total RNA was isolated from 4 X 10^7 HeLa cells using the RNeasy Mini kit (Qiagen) according to the protocol for isolation of cytoplasmic RNA from animal cells from the manufacturer's manual. The RNA was eluted in 60µl of water treated with diethyl pyrocarbonate (DEPC). Next, the absorbances of the samples were taken, and the concentration of the RNA was determined. The purity of the RNA was verified by optical density (OD) absorption ratio OD260 nm/OD280 nm between 1.80 and 2.1. 6µl of the RNA was also subjected to denaturing agarose gel electrophoresis and ethidium bromide staining to verify the integrity and size distribution, together with 4 µl RNA ladder, High Range (MBI Fermentas). This was performed according to manufacturer's protocol (Qiagen). The 18S and 28S bands appeared as sharp bands under UV.

3.1.12 Construction of complementary DNA (cDNA) library

A detailed outline of the protocol is shown in Figure 9. From the cytoplasmic total RNA, polyA$^+$ RNA was isolated using the Oligotex kit (Qiagen) and according to the mRNA spin-column protocol. 200µl of poly A$^+$ mRNA was eluted from the spin column, and all the isolated mRNA was used in the construction of the cDNA library.

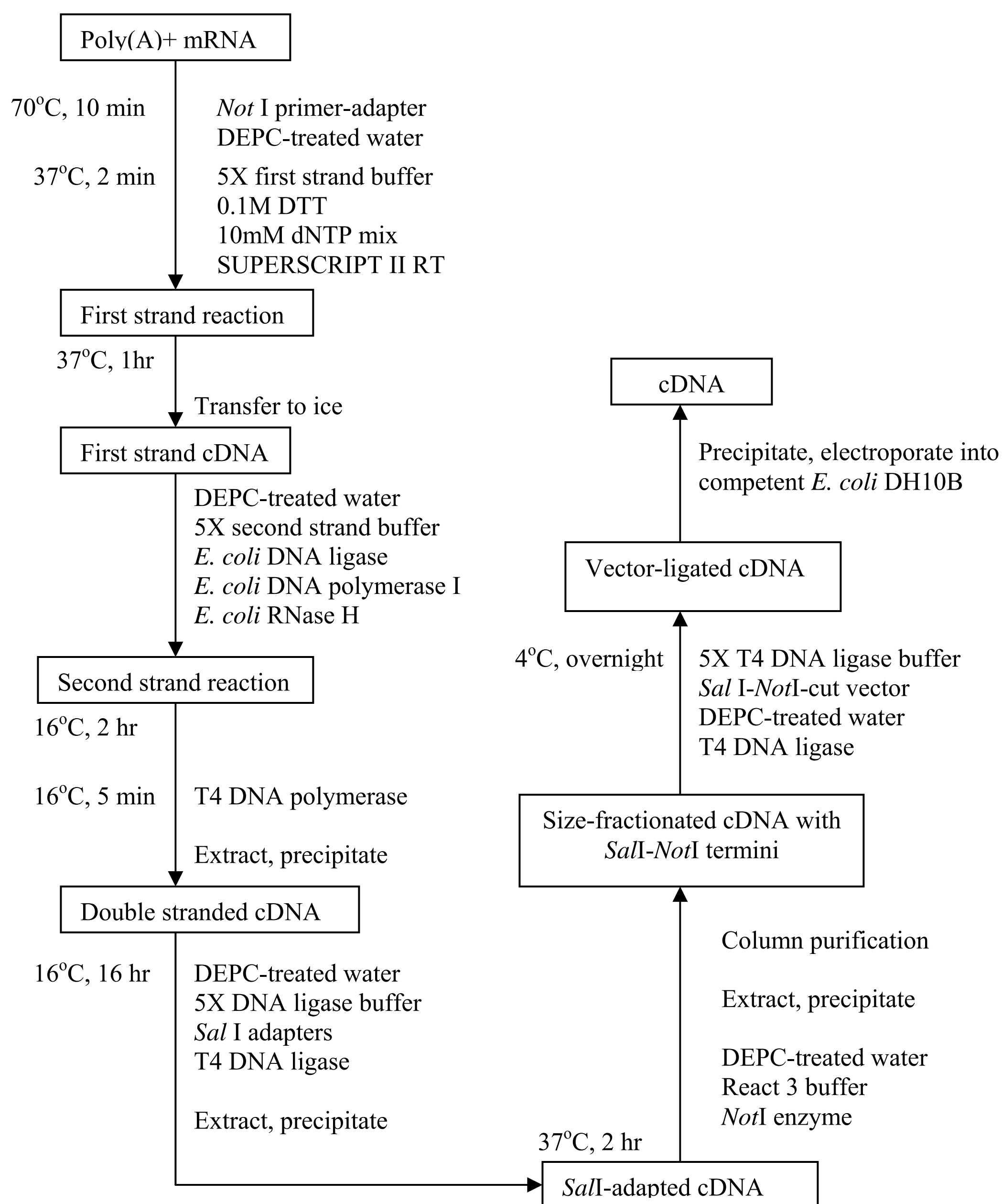

Figure 9: Detailed flow diagram of procedure of cDNA synthesis and cloning.

Construction of the cDNA library was carried out using the SUPERSCRIPT^{TM} plasmid system with GATEWAY^{TM} technology for cDNA synthesis and cloning (Invitrogen). This kit allows the directional cloning of the cDNA inserts in a specific

orientation relative to the transcriptional polarity of the original mRNAs. Directional cloning was made possible by the primer-adapter 5'- Pgactagttctagatcgcgagcggcc gccc$(T)_{15}$-3' in the first strand synthesis, and the ligation of *Sal*I adapters after the second strand synthesis. Size fractionation of the cDNA was not carried out; instead, purification was carried out using PCR column purification kit (Roche). This was because no radioactive dinucleotides were used in the protocol, and it would be difficult to analyze the yield in each fraction.

The 5' *Sal*I- and 3' *Not*I-digested cDNAs were then ligated into the yeast pACNX-N_{ub}IBC vectors in all three reading frames that have undergone restriction digestion with *Sal*I and *Not*I restriction enzymes. Large-scale ligation was carried out in a 1ml reaction mix consisting of 20µg of vectors in each reading frame, 200µl of 5X ligase buffer and 25µl of ligase at 4°C over the weekend. Electroporation was carried out using the competent *E. coli* DH10B cells. Following an one hour incubation at 37°C, the cells were plated onto Luria Bertani plate containing 10µg/ml of chloramphenicol; a total of 59 electroporations were performed. Dilutions of 10^{-3} 10^{-4} and 10^{-5} were carried out to find out the total number of transformants, and 20 colonies were picked from this dilution plate after incubation for 24 hours at 37°C. The plasmids were recovered by alkaline lysis and the DNA was then subjected to restriction digestion by *Hind*III and *Not*I to verify the presence of inserts. As for the 59 plates, 5 ml of LB was used to wash the cells off the agar plates, and the culture was subjected to large-scale preparation of plasmids using the alkaline lysis method and subjected to cesium chloride /ethidium bromide purification (Ausubel *et al*., 2006).

3.1.13 Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from 1 X 10^6 cells in each well of the 6-well plate as described above. 100ng of total RNA was used in the reverse transcription reaction (Applied Biosystems), with 1X reverse transcription buffer, 2.5µM Oligo-d(T)$_{16}$ primers, 0.5mM dNTPs, 5.5mM $MgCl_2$, 6 units of RNase inhibitor and 18.75 units of reverse transcriptase in a total of 15µl per reaction. The tubes were then placed into the PCR machine for 10 minutes at 25°C, followed by 60 minutes at 37°C and 5 minutes at 95°C. Real time PCR was performed using ABI Prism® 7700 (Applied Biosystems). Reactions were performed in 25µl volumes, containing 125nM of each primer, 1X SYBR® Green PCR Master Mix (Applied Biosystems), 5µl of reverse transcription reaction solution. Table 4 lists the primers used in the Real-time PCR. The experiments were performed in triplicates. The cycling parameters used for the Real time PCR were as follows: denaturation at 50°C for 2 minutes, 95°C for 10 minutes and subsequently 40 amplification cycles at 95°C for 15 seconds and 60°C for 60 seconds. A dissociation curve was carried out after each run to ensure that no unspecific products were formed.

Name of primer	Sequences	Length of amplicon
hGAPD Forward[1]	5'- ctctctgctcctcctgttcgac-3'	69bp
hGAPD Reverse[1]	5'- tgagcgatgtggctcggct-3'	
hHsp70B' Forward[2]	5'- ccccatcattgaggaggttg-3'	217bp
hHsp70B' Reverse[2]	5'- gaagcagaagaggatgaacc-3'	
hSkp1 Forward	5'- gcaaagagaaccagtggtgtga -3'	205bp
hSkp1 Reverse	5' - aggtttgggatctgtgctcaa -3'	

Table 4: List of primers used for real-time PCR.
Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPD) and hHsp70B' are as listed in the following journal: [1]Carraro *et al* 2004; [2]Parsian *et al*, 2000

The relative expression levels of hHsp70B' and hSkp1 mRNA in siRNA-treated samples and untreated samples were determined using the comparative threshold (Ct) method. The fold change in expression between siRNA treated and empty vector treated cells were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

3.2 Cell culture

Two cell lines were used in this study. One cell line was the human fibroblast HT1080HPRT^{-} that is lacking the gene for hypoxanthine-guanine phosphoribosyltransferase (HPRT). It was obtained from Sandra Pellegrini from the Department of Immunology, Institut Pasteur, Paris, France (Pellegrini *et al*., 1989). The other cell line was HeLa cells, a kind gift from Dr Seah Geok Teng's lab.

3.2.1 Maintenance of cell culture

The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma 1152, Numi preparation facility), supplemented with 10% (v/v) fetal calf serum (FCS) (Nalgene) in 75cm^2 tissue culture flask. When the cells were 90% confluent, the cells were trypsinised as follows: the medium was first removed. Next, 25ml of phosphate buffered saline (PBS; Numi preparation facility) was added to wash the cells, and then discarded. 3.5ml of 1X trypsin-EDTA solution (Sigma) was added to the flask, and incubated for 5 minutes at room temperature before tapping the flask to dislodge the adhesive cells. 10ml of medium was then added to neutralize the trypsin, and 11ml of it was removed and placed in a 15ml tube. To the medium remaining in the flask, 20ml of medium was used to top up, and the flask was placed back in the 37°C carbon dioxide

(CO_2; 5%) incubator. The 15ml tubes were centrifuged at 1,500 rpm for 5 minutes at 15°C. The pellet was resuspended in 2ml of 10% dimethylsulfoxide (DMSO) with FCS, and the solution transferred into a cryopresevation vial. These vials were then stored at -80°C or in liquid nitrogen.

3.2.2 Construction of stable C_{ub} cell lines

Stable C_{ub} cell lines (HT1080HPRT^{-}::Srb7-Cub-RGpt2) were made by lipofectamine transfection using LIPOFECTAMINE™ 2000 reagent (Invitrogen).

For transfection of C_{ub} fusion plasmid, HT1080HPRT^{-} cells were used. The day before the transfection, HT1080HPRT^{-} cells were trypsinized and plated onto 6-well plates such that it would be 90-95% confluent on the day of transfection. For each well of cells to be transfected, 0.5µg of plasmid DNA pcDNA3-Srb7-C_{ub}-RGpt2 was diluted in 100µl of serum-free DMEM in 1.5ml microtube. 3ml of LIPOFECTAMINE 2000 reagent was diluted in 100µl of serum-free DMEM in 1.5ml microtube for each well of cells. These two solutions were combined, and mixed gently before incubation at room temperature for 20 minutes. This step was for the formation of DNA-lipid complexes. The cells were washed once with 2ml of serum-free DMEM. For each transfection, 800µl of serum-free DMEM was added to each tube containing the lipid-DNA complexes and mixed gently. This diluted complex solution was then overlayed onto the washed cells, and the 6-well plate was incubated for 5 hours at 37°C in the 5% CO_2 incubator. 1ml of DMEM with 20% of FCS was added to the cells without removing the transfection mixture. The medium was replaced 24 hours following transfection.

48 hours after transfection, the cells were selected for gene expression of the zeocin marker in medium that contained 80mg/l of the zeocin. The cells in each well were trypsinized, and dilutions were carried out such that 10% of the cells were plated onto four 24-well plates. The incubation period was 14 days, and medium was replaced every 5 days or when necessary.

Single colonies were picked after the two weeks, and three cell lines were analyzed. Each single colony was trypsinised and transferred to one well of 6-well plates to grow to 90% confluence before testing for phenotype.

3.2.3 Construction of stable N_{ub} cell lines

Stable N_{ub} cell lines were made by lipofectamine transfection similar to the construction of stable C_{ub} cell lines. However, there were two key differences. N_{ub} fusion plasmids were transfected into the cells that had been stably transformed with C_{ub} fusion plasmids (HT1080HPRT$^-$::Srb7-C_{ub}-RGpt2), and the cells were selected for the expression of neomycin phosphotransferase encoded by the N_{ub} fusion vector. The medium used for the selection of the N_{ub} cell lines was medium containing G418 sulfate (400mg/l) (USB, Amersham International). The cells were diluted such that there were three 24-well plates with 90%, 10% and 1% of the cells on each plate. The incubation time was about 14 days, and the medium was replaced every 5 days or when necessary. Single colonies were picked, and two stable cell lines of each construct were analyzed for their phenotype.

3.2.4 Analysis of phenotype

For each C_{ub} cell line construct, 10% of the cells, upon trypsinization, were placed in each of the three wells of a 6-well plate. To one well, 2ml of DMEM with 10% FCS was added. The second well was supplemented with 2ml of hypoxanthine/aminopterin/thymidine (HAT) medium (Roche, Boehringer Mannheim). As for the last well, 2ml of medium with 6-thioguanine (6TG; Sigma) (15mg/l) and 80mg/l of zeocin was added. The solutions were changed every three days. The procedures for analysis of phenotype in N_{ub} constructs were similar except for the addition of G418 (400mg/l) to medium containing HAT in the second well. When the cells were 90% confluent, trypsinization was carried out, and 90% of the cells in each well were removed. This step was to ensure that dead cells were removed. The remaining cells were left to grow to 90% confluent before photographs were taken to analyze their phenotype.

3.2.5 Transfection of plasmids and heat induction of HeLa cells for Real time PCR experiment

The experiment was carried out in 6-well plates. 1µg of plasmid was transfected into 90% confluent HeLa cells using 3µl of Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Heat induction was carried out at 45°C for 15 minutes, and subjecting the cells to recovery at 37°C for an hour (Parisan *et al.*, 2000) before harvesting them (as above).

3.3 Western blot

3.3.1 Preparation of samples and acrylamide gels

Separating gels with 12% acrylamide concentrations were used in this study, while a 4% stacking gel was consistently used (Table 5). Formulations of SDS-polyacrylamide separating and stacking gels are listed in Table 5. Recipes are sufficient for the preparation of 1 slab minigels (0.75 mm thick and 100 by 70mm^2), and the components were mixed in the order shown. Polymerization would begin as soon as N,N,N',N'-tetramethlethylene-diamine (TEMED) had been added.

Separating gels	**12%**
3.3 ml	4.0 ml
4.0 ml	3.3 ml
2.5 ml	2.5 ml
100 µl	100 µl
100 µl	100 µl
4 µl	4 µl
10 ml	10 ml
Stacking gels	**4%**
Sterile distilled water	3.0 ml
30% acrylamide mix	0.65 ml
1.0 M Tris Cl (pH6.8)	1.25 ml
10% SDS	50 µl
10% ammonium persulfate	50 µl
TEMED	5 µl
Total (for 1 gel)	5 ml

Table 5. Solutions for preparing gels for SDS-PAGE.

3.3.2 Electrophoresis and Western blot

50µl of PBS and 50µl of Laemmli buffer (Bio-Rad Laboratories) were added to resuspend the pelleted cells, and no heating of the samples at 100°C was necessary. 15µl of each sample was separately loaded into the sample wells, and 5µl of the molecular marker standard - Kaleidoscope Prestained Standards (Bio-Rad Laboratories) – was heated for 1 minute at 40°C before loading onto the gel. Electrophoresis was carried out

at 100V at room temperature. The voltage was increased to 150V when the samples had entered the separating gel and monitored till the dye reached the end of the gel. At the end of the run, the glass plates were removed from the gel stand, and the plates were gently pried apart.

The next step was to transfer the separated proteins onto a nitrocellulose membrane using a Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories). A sandwich was assembled by placing 4 pieces of filter papers presoaked in 1X transfer buffer onto the platinum anode, followed by the pre-wet nitrocellulose membrane, the gel and 4 more pieces of soaked filter paper. Appropriate markings were made to indicate the relative positions between the gel and the membrane, and air bubbles were carefully removed from between each layer. After the cathode and safety cover were placed onto the stack, the electrophoretic transfer was performed at 0.2A for 60 minutes.

The blotted membrane was then placed in blocking buffer that contained 5% non-fat dried milk in 100ml deionised water with 10 mM Tris HCl (pH7), and soaked in this solution for 1 hour at room temperature on an orbital shaker. This was to block non-specific binding sites. The next step was to incubate the membrane with the primary antibody, the anti-haemagglutinin (HA) antibody (BabCO, Freiburg, Germany). The dilution was 1:10000 in 5% non-fat milk in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween-20). The incubation was overnight, with the tray placed on an orbital shaker. After three 15-minute washes with 1% non-fat milk to remove any unbound primary antibody, incubation with the secondary antibody - horseradish peroxidase-coupled anti-mouse antibody (Bio-Rad, München, Germany) - was carried out next. This incubation

was for 2 hours at room temperature on an orbital shaker, and the dilution of the secondary antibody was 1:4,000 in 5% non-fat milk in TBST. Following this, three washes of 15 minutes each were performed using TBST. Excess wash buffer from the washed membrane was drained, and the nitrocellulose membrane was placed protein side-up on a clean surface.

ECL Plus (Amersham Pharmacia Biotech) system was used to detect the proteins, and the detection reagents were prepared by mixing solution A and B in the ratio of 40:1. The mixed detection reagent was then pipetted onto the entire surface of the membrane, and allowed to incubate for 1 minute. Any excess detection reagent was drained off by holding the membrane gently with forceps. SaranWrap was subsequently used to wrap up the blot while taking care to smooth out any air bubbles, and the wrapped blot was placed protein side-up onto a X-ray film cassette. This next step was carried out in a dark room using red safe lights. A sheet of autoradiography film was placed on top of the membrane, and the cassette was closed to expose for 5 minutes. The film was developed at the darkroom at the Clinical Research Center (National University of Singapore). Based on the appearance of the first film, estimations were made to continue the second exposure of the second piece of film.

3.4 Working with *Saccharomyces cerevisiae*

3.4.1 Competent yeast cells and transformation

The yeast strain used was NLY2 (MATα, *gal4⁻, gal80⁻, ade2⁻, ura3-52, leu2-1, his3Δ200, trp1::hisG, lys2::hisG*) (Saha *et al.*, 1993).Yeast cells were made competent using the lithium acetate method as stated in Ausubel *et al* (2006). Cells were kept at

4°C for a maximum of two weeks. Small-scale transformation of plasmids into the competent yeast cells were as follows: 0.5µg of plasmid was added to 2µl of fish sperm carrier DNA, followed by 10µl of competent cells (NLY2 with pCup1-hSkp1-C_{ub}-RUra3) and 50µl 40% PEG solution. The solutions were vortexed to ensure mixing, and incubated at 28°C for 1 hour. Subsequently, the tubes were heat-shocked in a 42°C water bath for 15 minutes. Next, the cells were pelleted by spinning at 7000rpm for 1 minute; the pellet was resuspended in 20µl of sterile water, and plated onto a suitable selection plate.

The screening of cDNA for interacting partners of hSkp1-C_{ub}-RUra3 required large-scaled transformation. Here, 200µl of cDNA library fused downstream of N_{ub} was added to 100µl of fish sperm carrier DNA. 200µl of competent cells (NLY2 + hSkp1-C_{ub}-RUra3) were added next, followed by 400µl of 40% PEG. The cells were vortexed before incubation at 28°C for an hour, and subsequently subjected to heat shock. The cells were then pelleted by spinning at 7,000rpm for 1 minute; the pellet was resuspended in 4ml of sterile water, and spread on 7 plates plate lacking tryptophan and leucine ($W^{-}L^{-}$, termed WL) and containing 5-fluororotic acid (5-FOA) ($F^{-}W^{-}L^{-}$; denoted FWL plate) and 7 FWL plates containing 100mM copper sulphate, labeled as 100FWL. The plates were incubated at 28°C for 3 to 10 days, and were checked for colonies regularly.

3.4.2 Plasmid recovery and electroporation

The plasmids were recovered from yeast cells using the phenol-chloroform extraction method (Ausubel *et al*, 2006). The plasmids were then introduced into *E. coli*

DH10B cells through electroporation (BioRad, MicroPulser^{TM}) using 4µl of plasmid with 40µl of *E. coli* cells. After recovery for 1 hour at 37°C, the cells were plated onto selective media LB plates containing chloramphenicol. Alkaline lysis miniprep was then carried out to isolate the plasmids from *E. coli* cells as described above.

3.4.3 Droplet assay

In a 96-well plate format, ten-fold serial dilutions of yeast cells were carried out to 10^{-5} dilutions using a multi-pippetter. The total volume per well was 100µl. 5µl of the cells were plated onto the following plates: plates depleted of tryptophan and leucine (WL), uracil depleted plates ($U^{-}W^{-}L^{-}$; termed UWL) and the WL plates with 5-fluoroorotic acid (5-FOA) added (labeled as FWL). Two other plates each had 100µM copper sulphate ($CuSO_4$) added; as such they are labeled as 100FWL and 100UWL plates. These plates were incubated at 28°C for 3 days before an analysis of the growth was made.

CHAPTER 4

RESULTS

4. Results

4.1 Cloning of inserts into yeast vectors and mammalian vectors

Polymerase chain reaction (PCR) was carried out to obtain the open reading frame (ORF) of hSKP1 and hSRB7, and the PCR products were subjected to electrophoresis to analyze the amplified products. The expected sizes of each product are shown in Table 1, and Figure 10a shows the PCR sample after electrophoresis. Restriction digestions of PCR products and of plasmids were carried out, and ligation was performed. Subsequently, DNA minipreparations by alkaline lysis were carried out to recover the plasmids, and restriction digestion as well as gel electrophoresis was carried out to check if the transformants carried the insert of the right size. Figure 10b shows the inserts for mammalian vectors cDNA3-hSrb7-C_{ub}-RGpt2 and cDNA3-N_{ub}I2-hSkp1. The cloning of pCup1-hSrb7-C_{ub}-RUra3 and pACNX-N_{ub}I2-hSkp1 was also successful. Next, DNA sequencing was performed to verify that the inserts were cloned in the right reading frame and there were no mutations. No mutations were observed in hSkp1 and hSrb7 and the inserts were cloned in frame with N_{ub} and C_{ub} respectively for both yeast and mammalian vectors (Refer to Appedice 3 and 4 for sequencing results). Cloning of pACNX-N_{ub}I2-hSgt1 was also successful.

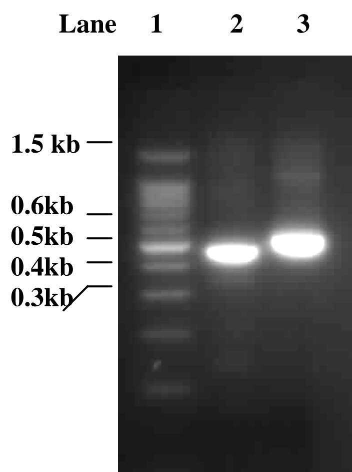

Figure 10a: Analysis of hSrb7 and hSkp1 PCR products.
The DNA bands were stained with ethidium bromide, and viewed under UV. Lane 1 shows the 100bp DNA ladder, Lane 2 is the PCR product of the ORF of hSrb7, and Lane 3 is the PCR product of the ORF of hSkp1. The sizes of hSrb7 and hSkp1 are 435bp and 492bp respectively.

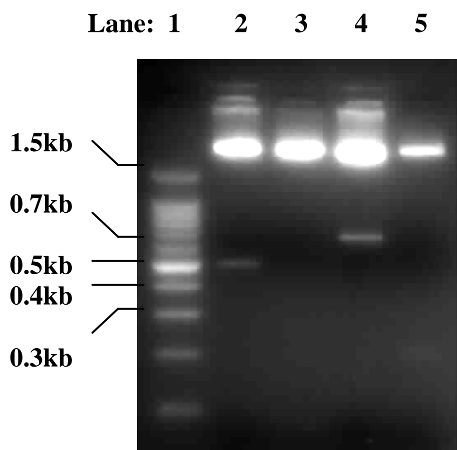

Figure 10b: Analysis of pcDNA3-hSrb7-C_{ub}-RGpt2 and pcDNA3-N_{ub}I2-hSkp1 constructs.
The DNA bands were stained with ethidium bromide and viewed under UV. Lane 1 shows the 100bp DNA ladder. Lane 2 is the pcDNA3-Srb7-C_{ub}-RGpt2 plasmid that has been subjected to restriction digestion with *Hind*III and *Xho*I, and the expected sizes are 5701 bp (plasmid back bone) and 487 bp (insert). Lane 3 is the negative control pcDNA3-C_{ub}-RGpt2 vector that has been cut with *Hind*III and *Xho*I. In lane 4, pcDNA3-N_{ub}-Skp1 has been digested with enzymes *Hind*III and *Xba*I, and the expected sizes are 5352bp (plasmid back bone) and 663bp (insert). As for lane 5, it is the empty vector pcDNA3-N_{ub} that has been subjected to restriction digestion with *Hind*III and *Xba*I.

4.2 Interaction of hSrb7-C_{ub}-RUra3 and N_{ub}-hSkp1 in yeast

PCup-hSrb7-C_{ub}-RUra314 was transformed into the yeast strain NLY2, and transformants were selected on plates lacking tryptophan (W^-, designated W). pACNX-N_{ub}-hSkp1 was then introduced into the above yeast strain, and selected on plates lacking tryptophan and leucine (W^-L^-, termed WL). The strength of the interaction between the

two fusion proteins was quantified using ten-fold serial dilutions of cells coexpressing the fusion proteins. The serial dilutions were dropped onto WL plates, media lacking uracil, tryptophan and leucine ($U^-W^-L^-$, termed UWL), media lacking uracil, tryptophan and leucine and containing 100 µM copper sulphate ($CuSO_4$) (100 UWL), media containing 5-FOA and lacking tryptophan and leucine ($F^-W^-L^-$, termed FWL) and media containing 100µM $CuSO_4$, 5-FOA and lacking tryptophan and leucine (100 FWL). Figure 11 shows the results obtained, co-transformants expressing N_{ub}-hSkp1 and hSrb7-C_{ub}-RUra3 proteins did not grow well in media lacking uracil, but survived on media containing 5-FOA. This indicated that protein-protein interaction between full-length hSrb7 and hSkp1 was observed using the yeast split-ubiquitin system.

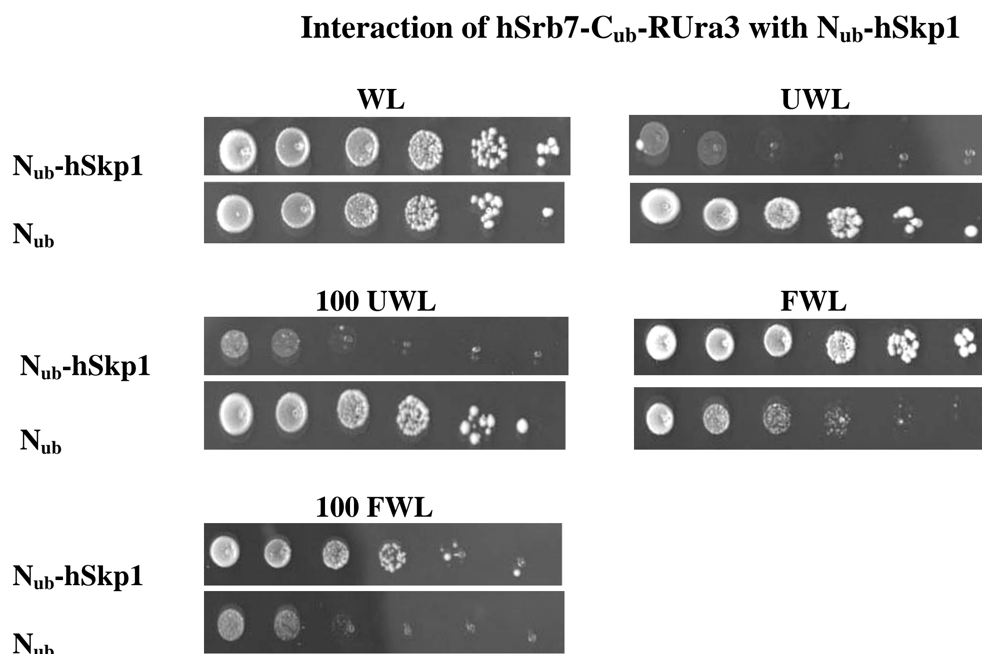

Figure 11: Full-length hSrb7p interacts with full-length hSkp1p in *S. cerevisiae*.
The ten-fold serial dilution of cells co-expressing the hSrb7-C_{ub}-RUra3 and N_{ub}-hSkp1 or N_{ub} are shown above, with the serial dilutions in decreasing order from left (10^0) to right 10^{-5}). The cells were spotted on media lacking tryptophan and leucine (termed WL; top left), additionally lacking uracil (UWL; top right), additionally lacking uracil with 100 µM $CuSO_4$ (100UWL; middle left), containing 5-FOA (FWL; middle right) and containing 5-FOA with 100 µM $CuSO_4$ (100 FWL; bottom left). The cells expressing the interacting proteins grew less well on UWL and 100 UWL plates, and displayed a stronger resistance to 5-FOA than the empty vector N_{ub}.

4.3 Construction of HT1080HPRT$^-$::hSrb7-C_{ub}RGpt2 cell line

The hSrb7-C_{ub}-RGpt2 plasmid was transfected into HT1080HPRT$^-$ cell, and stable cell lines were selected by the zeocin resistance selection marker on the plasmid. After two weeks of incubation, sixteen independent cell lines (i.e. single colonies) containing hSrb7-C_{ub}-RGpt2 were picked and an analysis of their phenotype was performed in the two different mediums: medium containing HAT, and medium containing 6-TG and zeocin. After two weeks of incubation, it was found that of the sixteen independent cell lines, two were HAT resistant and 6-TG sensitive, whereas the remaining 14 were HAT sensitive and 6-TG resistant. The expected phenotype of cell lines expressing the hSrb7-C_{ub}-RGpt2 fusion was HAT resistant and 6-TG sensitive, which meant that two cell lines were stably transfected with hSrb7-C_{ub}-RGpt2.

4.4 Interaction of hSkp1 with hSrb7 in the mammalian split-ubiquitin system

N_{ub}-hSkp1 was co-transfected into one of the two stable cell lines, and two independent cell lines were analyzed for its phenotype in medium containing HAT and G418, and in medium containing 6-TG and zeocin. Figure 12 shows the phenotype of cells observed when transfected with N_{ub} and N_{ub}-hSkp1, and this indicated that interaction between N_{ub}-hSkp1 and hSrb7-C_{ub}-RGpt2 was detected using the mammalian split-ubiquitin system.

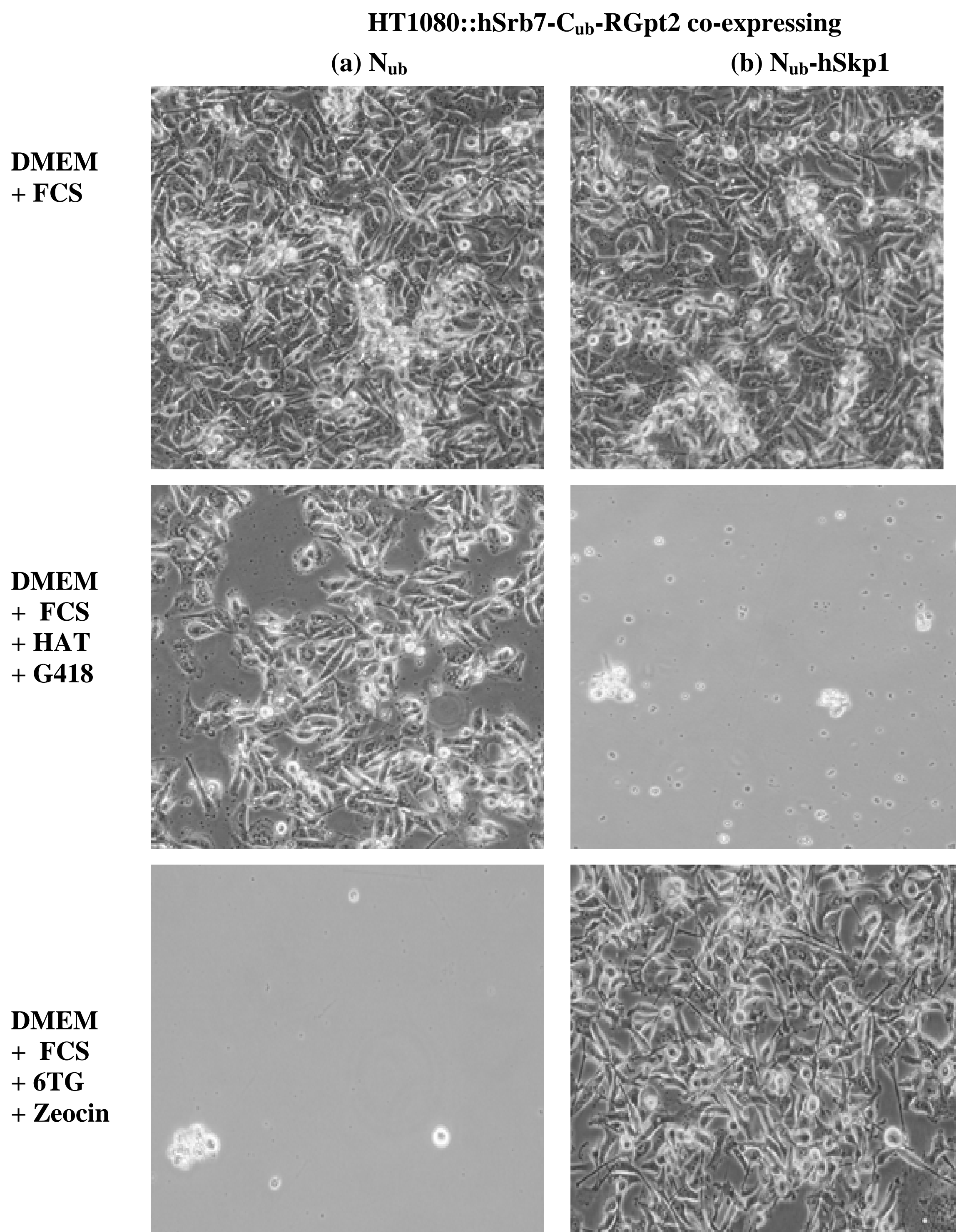

Figure 12: Interaction between hSrb7-C_{ub}-RGpt2 and N_{ub}-hSkp1 was observed using the mammalian split-ubiquitin system.
Left column: An HT1080HPRT$^-$ cell line coexpressing hSrb7-C_{ub}-RGpt2 and N_{ub} was grown in DMEM +10% FCS (first line), in DMEM +10% +HAT +G418 (second line) or in DMEM +10%FCS +6-TG +Zeocin (third line). This cell line was observed to be HAT^R and 6TG^S. Right column: HT1080HPRT$^-$ cell line coexpressing hSrb7-C_{ub}-RGpt2 and N_{ub}-hSkp1 grown in the different selection media as described above. This cell line was observed to be HAT^S and 6TG^R, indicating that hSrb7-C_{ub}-RGpt2 and N_{ub}-hSkp1 interacts.

4.5 Analysis of knock-down of hSkp1 on inducible Hsp70B' transcripts

The 19-nucleotide target against the 3' UTR region of the hSkp1 mRNA was determined. Based on the pSuper RNAi system, two 60-nucleotide long oligo were designed. Upon annealing of the two oligos, the double-stranded oligo was ligated into the pSuper vector that was cleaved with *Hind*III and *Sal*I. Next, the vector was transformed into *E. coli* and alkaline lysis was performed. Figure 13 shows the results restriction digestion of vector and gel electrophoresis.

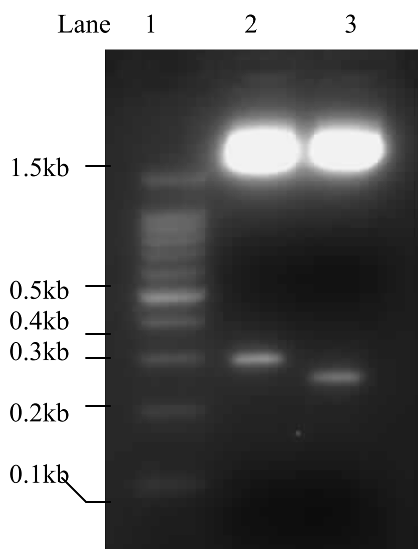

Figure 13: Analysis of pSuper-681-hSkp1 siRNA construct after restriction digestion and gel electrophoresis.
The DNA bands have been stained with ethidium bromide and were viewed under UV. Lane 1 shows the 100bp DNA ladder. In lane 2, the plasmid pSuper-681-Skp1 has been subjected to restriction digestion with *Eco*RI and *Xho*I to give two DNA bands of 4353bp (plasmid back-bone) and 287bp (insert). This indicated that the cloning of the target sequence into the vector was successful. Lane 3 shows restriction digestion product of empty vector pSuper with the same enzymes. The expected size is 4353bp (plasmid back bone) and 248bp.

Knock-down experiment of hSkp1 was performed, and Western blot with anti-hSkp1 antibody was used to verify the knockdown of hSkp1 in HeLa cells (Figure 14).

Figure 14: Analysis of the effects of hSkp1 siRNA on hSkp1p in HeLa cells.
The Western blot was performed using antibodies against hSkp1p, and the expected size of the protein was 19kDa. Lane 1 shows the untransfected HeLa cells. In lanes 2, 3 and 4, HeLa cells have been transfected with 1ug, 2ug and 4ug of pSuper-hSkp1 681 respectively.

A different approach, the two-step reverse transcription polymerase chain reaction (RT-PCR) was carried out to verify the knockdown of hSkp1. Reverse transcription was first carried out, and real-time PCR using SYBR-Green was performed next. The expected amplicon size was 206 base pairs. Relative expression levels of hSkp1 mRNA in siRNA-treated samples and untreated samples were determined using the comparative threshold (C_T) method. The fold change in the RNA expression between siRNA treated and untreated cells were calculated using the $2^{-\Delta\Delta CT}$ method, with the human glyceraldehyde-3-phosphate dehydrogenase (GAPD) as an endogenous reference. The data of the quantitative analysis is shown in Table 6, and Figure 15a shows the results as a chart.

From Figure 15a, it was observed that hSkp1 siRNA was able to reduce the hSkp1 transcript levels by 2-fold as compared to untreated cells. The induction of cells with heat had no effects on the mRNA levels of hSkp1, and any differences observed in the levels of hSkp1 transcripts was due to the treatment of cells with hSkp1 siRNA. For the same samples, the relative quantitation of Hsp70B' RNA was also analyzed. This was to find out the effects of hSkp1 siRNA and heat induction on its mRNA. The results were reported in Table 7, and in Figure 15b as a chart.

Sample	hSkp1 Average C_T	GAPD Average C_T	ΔC_T hSkp1-GAPD[a]	$\Delta\Delta C_T$ C_T - $C_{T, \text{Untreated NHS}}$[b]	hSkp1 Rel to Untreated NHS[c]
Untreated, NHS	25.92 ± 0.17	17.39 ± 0.03	8.53 ± 0.17	0 ± 0.17	1.0 (0.88, 1.12)
Untreated, HS	25.63 ± 0.00	17.11 ± 0.08	8.52 ± 0.08	-0.01 ± 0.08	1.0 (0.95, 1.06)
Treated NHS	27.00 ± 0.12	17.40 ± 0.09	9.60 ± 0.14	1.07 ± 0.14	0.47 (0.43, 0.52)
Treated, HS	26.79 ± 0.15	17.24 ± 0.01	9.55 ± 0.15	1.02 ± 0.15	0.49 (0.44, 0.54)

Table 6: Relative quantitation of hSkp1 mRNA using the comparative C_T method.
Untreated cells refer to HeLa cells that were not treated with hSkp1 siRNA, but with the empty pSuper vector. HeLa cells that have been treated with siRNA against hSkp1 were labeled 'Treated'. NHS: Non heat-shocked cells; HS: cells induced with heat at 42°C for 15 min. After an hour of recovery at 37°C, the cells were then harvest for the isolation of total RNA.

a. The ΔC_T value is determined by subtracting the average GAPD C_T value from the average hSkp1 C_T value. The standard deviation of the difference is calculated from the standard deviations of the hSkp1 and GAPD values, according to the following formula s $\sqrt{s_1^2 + s_2^2}$ where s = standard deviation.
b. The calculation of $\Delta\Delta C_T$ involves subtraction by the ΔC_T calibrator value. This is the subtraction of an arbitrary constant, so the standard deviation of $\Delta\Delta C_T$ is the same as the standard deviation of the ΔC_T value.
c. The range given for Skp1 relative to untreated NHS is determined by evaluating the expression: $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T + s$ and $\Delta\Delta C_T - s$, where s = the standard deviation of the $\Delta\Delta C_T$ value.

Sample	Hsp70B' Average C_T	GAPD Average C_T	ΔC_T Hsp70B'-GAPD[a]	$\Delta\Delta$ C_T C_T - $C_{T, \text{Untreated NHS}}$[b]	Hsp70B' Rel to Untreated NHS[c]
Untreated, NHS	29.95 ± 0.00	17.39 ± 0.03	12.56 ± 0.03	0 ± 0.03	1.0 (0.98, 1.02)
Untreated, HS	21.60 ± 0.00	17.11 ± 0.08	4.49 ± 0.08	-8.07 ± 0.08	268.0 (254, 284)
Treated, NHS	29.56 ± 0.08	17.40 ± 0.09	12.16 ± 0.12	-0.4 ± 0.12	1.3 (1.21, 1.43)
Treated, HS	23.88 ± 1.20	17.24 ± 0.01	6.64 ± 1.20	-5.92 ± 1.2	60.5 (26.9, 139.1)

Table 7: Relative quantitation of Hsp70B' mRNA using the comparative C_T method.
Untreated cells refer to HeLa cells that were not treated with hSkp1 siRNA, but with the empty pSuper vector. HeLa cells that have been treated with siRNA against hSkp1 were labeled 'treated'. NHS: Non heat-shocked cells; HS: cells induced with heat at 42°C for 15 min. After an hour of recovery at 37°C, the cells were then harvest for the isolation of total RNA.

a. The ΔC_T value is determined by subtracting the average GAPD C_T value from the average Hsp70B' C_T value. The standard deviation of the difference is calculated from the standard deviations of the Hsp70B' and GAPD values, according to the following formula s $\sqrt{s_1^2 + s_2^2}$ where s = standard deviation.
b. The calculation of $\Delta\Delta C_T$ involves subtraction by the ΔC_T calibrator value. This is the subtraction of an arbitrary constant, so the standard deviation of $\Delta\Delta C_T$ is the same as the standard deviation of the ΔC_T value.
c. The range given for Hsp70B' relative to untreated NHS is determined by evaluating the expression: $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T$ + s and $\Delta\Delta C_T$ – s, where s = the standard deviation of the $\Delta\Delta C_T$ value.

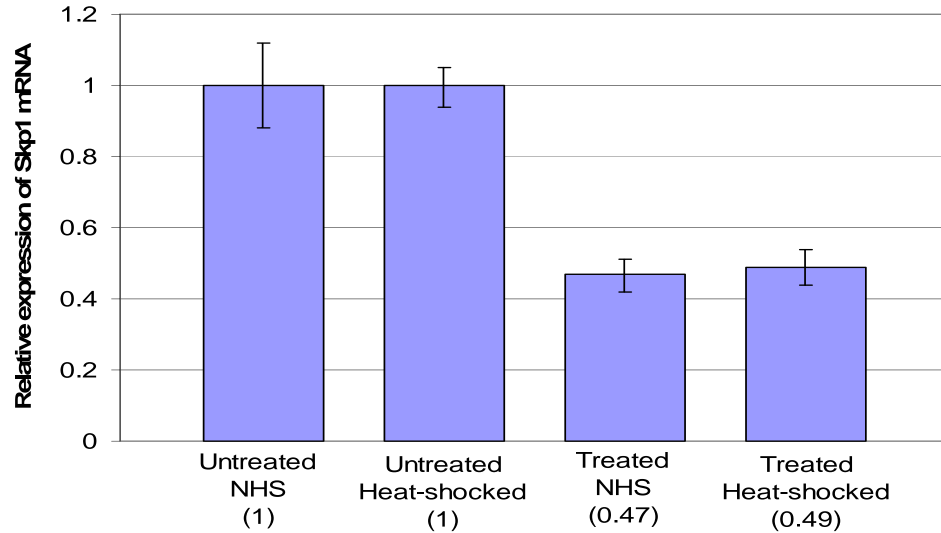

Figure 15a: Reduction in hSkp1 RNA levels was observed using the hSkp1 siRNA. The bar chart was constructed from the data stated in Table 6. Total RNA was isolated from the cells, and hSkp1 transcript levels were analyzed by reverse transcription and real-time PCR.

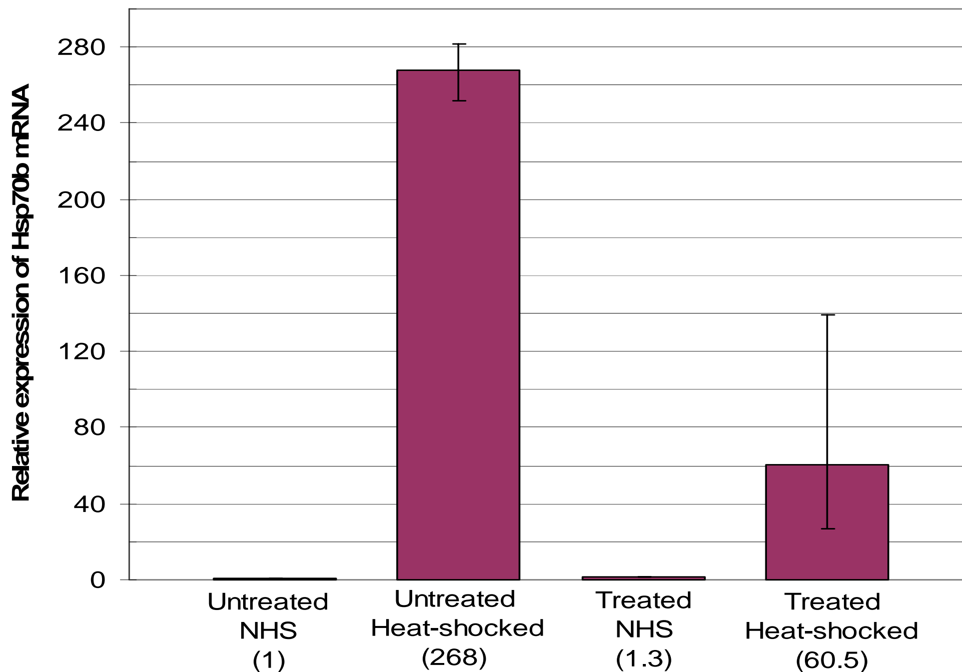

Figure 15b. Reduction in hSkp1 RNA levels reduced the activated Hsp70B' RNA. The bar chart was constructed from the data stated in Table 7. Total RNA was isolated from cells and Hsp70B' transcript levels were analyzed by reverse transcription and real-time PCR.

Untreated cells refer to HeLa cells that were not treated with hSkp1 siRNA, but with the empty pSuper vector. HeLa cells that have been treated with siRNA against hSkp1 were labeled as 'Treated'. The relative expression level of Hsp70B' RNA in each sample, with respect to untreated NHS sample, is provided in the parathesis. NHS: Non heat-shocked cells; HS: cells induced with heat at 42°C for 15 min. Error bars indicate one standard deviation.

From Table 7 and Figure 15b, it was observed in HeLa cells that were not treated with hSkp1 siRNA ('Untreated'), there was a 268-fold increase in the transcripts of Hsp70B' upon heat induction as compared non-heat shocked cells. This was as expected. The Hsp70B' mRNA of NHS cells treated with hSkp1 siRNA ('Treated, NHS') were similar to the untreated NHS cells.

However, for the sample that was subjected to heat-induction and treatment with hSkp1 siRNA (Figure 15b, designated 'Treated, heat-shocked'), there was only a 60-fold increase in the Hsp70B' transcript levels as compared to the untreated NHS sample. These results indicated that there was a significant decrease of Hsp70B' mRNA as compared to the heat-induced sample that had the wild-type levels of hSkp1 transcript ('untreated, heat-shocked'). These results suggested hSkp1p was required for maximal induction of Hsp70B' transcripts.

4.6 Construction of a human cDNA library fused to N_{ub}

Total RNA was isolated from four 75 cm^2 flask of HeLa cells, and some of the total RNA from each flask was subjected to denaturing agarose gel electrophoresis and ethidium bromide staining to observe the integrity of the 18S and 28S rRNA (Figure 16).

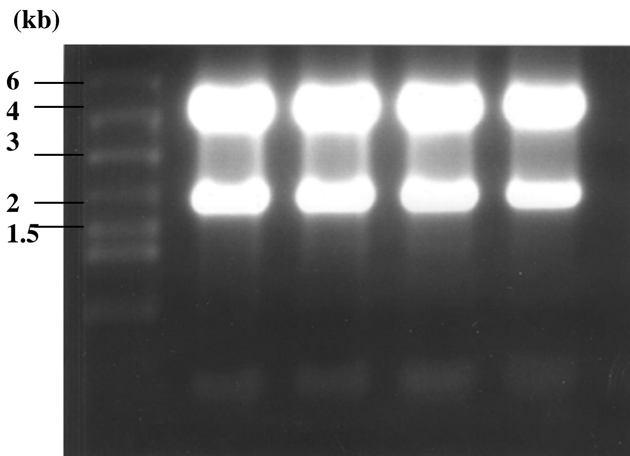

Figure 16: Formaldehyde agarose gel of total RNA isolated from four flasks of HeLa cells using the RNeasy Kit.
The rRNA bands were stained with ethidium bromide and viewed under UV. The sizes for the human 18S rRNA is 1.9 kb, and the 28S rRNA is 5.0 kb. The sizes of the RNA ladder, High Range, are as listed in the left hand side.

Next, mRNA isolation was performed, followed by the first strand synthesis using *Not*I primer-adapter in the reverse transcription reaction. The second strand reaction was then carried out. The last step in the cDNA synthesis procedure was to ensure that the termini of the cDNA were blunt-end by using the T4 DNA polymerase. *Sal*I adapters were added to the blunt-end double stranded cDNA, and the *Sal*I-adapted cDNA was subjected to restriction digestion with *Not*I enzyme. Purification was performed next to remove the restriction enzymes and any unligated adapters, and the purified cDNA with *Sal*I-*Not*I termini were then ligated to the *Sal*I-*Not*I digested pACNX-N_{ub} vectors in the three reading frames. A small-scale transformation was carried out to ascertain the number of clones, and cells were diluted 10^{-3}, 10^{-4} and 10^{-5} times in LB and plated. A total of 40 colonies were obtained on the sector plated with transformants diluted 10^{-4} times, and therefore the estimation of the total number of independent colonies was 4 x 10^5. DNA minipreparations of twenty colonies were performed to find out the proportion of vectors with cDNA inserts and six plasmids of a total of twenty plasmids were observed to contain an insert. The cDNA insert sizes ranged from 300bp to 1000bp. Therefore it was estimated that 30% of the 4 x 10^5 colonies had an insert (1.2 x 10^5). The remainder of the ligation sample was concentrated by ethanol precipitation, and electroporation was carried out to introduce the plasmids into competent DH10B *Escherichia coli* cells.

4.7 Screening of a human cDNA library fused to N_{ub} using hSkp1-C_{ub}-RUra3 as a bait

hSkp1 was cloned into the yeast vector Pcup1-C_{ub}-RUra314 upstream of the C_{ub}. Following the C_{ub} is the uracil gene whereby the first amino acid has been replaced by an

arginine. Thus for the fusion C_{ub}-RUra3, uracil prototrophy was an indication that the plasmid was successfully transformed into the yeast, and that it was expressed and functional. This plasmid contained a *TRP1* gene, giving rise to tryptophan prototrophy when transformed into yeast cells. Therefore, yeast cells transformed with the C_{ub} plasmid were plated onto media lacking tryptophan (W^-).

ySgt1p (suppressor of G2 allele of Skp1) was shown to interact with ySkp1p *in vitro* and *in vivo* (Kitagawa *et al*., 1999), and here, the human homologs were used to see if the protein pair interact in *S. cerevisiae*. PCR and cloning were performed to clone the ORF of hSGT1 into the yeast vector pACNX-N_{ub} to obtain the fusion protein of N_{ub}-hSgt1. As hSkp1p has been shown to be capable of forming dimers (Ng *et al.,* 1998), N_{ub}-hSkp1 was also tested in the protein-protein interaction assay (Figure 17). Interaction between N_{ub}-hSgt1 and N_{ub}-hSkp1 with hSkp1-C_{ub}-RUra3 was observed based on that the poor growth of the respective double transformants on plates lacking uracil, and growth on plates containing 5-FOA.

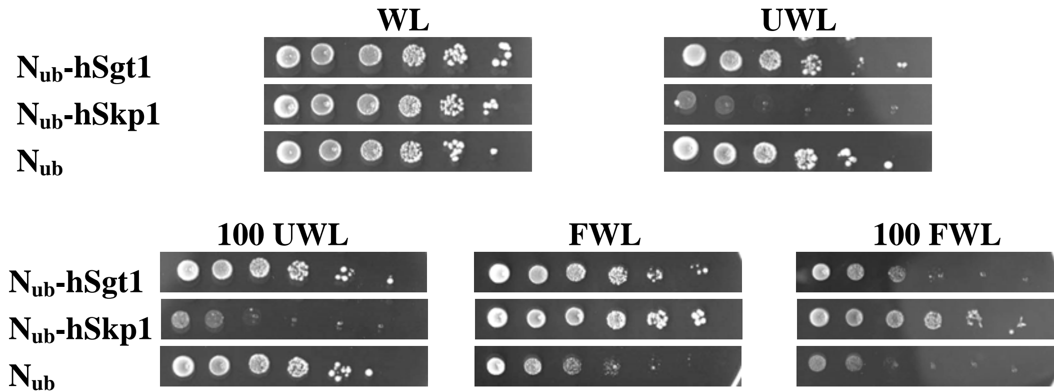

Figure 17: N_{ub}-hSgt1 and N_{ub}-hSkp1 interacts with hSkp1-C_{ub}-RUra3 in *S. cerevisiae*.
The ten-fold serial dilution of cells coexpressing hSkp1-C_{ub}-RUra3 and N_{ub}-hSkp1 / N_{ub}-hSgt1 / N_{ub} are shown above, with the serial dilutions in decreasing order from left (10^0) to right 10^{-5}). The cells were spotted on media lacking tryptophan and leucine (termed WL; top left), additionally lacking uracil (UWL; top right), additionally lacking uracil with 100 µM $CuSO_4$ (100 UWL; middle left), containing 5-FOA (FWL; middle right) and containing 5-FOA with 100 µM $CuSO_4$ (100 FWL; bottom left). The cells expressing the interacting proteins grew less well on UWL and 100 UWL plates, and displayed a stronger resistance to 5-FOA than the empty vector N_{ub}.

Since hSkp1-C_{ub}-RUra3 construct was shown to be enzymatically active, the next step was to screen the N_{ub}-cDNA library fusion proteins to isolate interacting partners of hSkp1. The hSkp1-C_{ub}-RUra3 plasmid was transformed into competent NLY2 cells, and the cells containing the C_{ub} fusion vector were then transformed with the N_{ub}-cDNA fusion plasmids to test for protein-protein interactions. WL plates with the addition of the drug 5-fluoro-orotic acid (5-FOA; termed FWL), and such plates with the addition of 100 µM $CuSO_4$ (100 FWL) were used to select for yeast cells containing both the interacting C_{ub} and N_{ub} protein in the yeast split-ubiquitin assay. As the plasmid containing the C_{ub}-RUra3 gene was driven by the CUP1 promoter, increased expression of the fusion protein was induced when $CuSO_4$ was added. Cells were also plated onto WL plates as a positive control to find out the total number of colonies that were successfully transformed. The

plating was carried out in dilutions of 10^{-3}, 10^{-4} and 10^{-5}, and 50 colonies were obtained for 10^{-5} dilution. This indicated that the primary number of independent transformants was 5 x 10^{6}. A total of 14 colonies were obtained on the FWL and the 100 FWL plates after ten day incubation at 28°C.

These 14 colonies were then isolated from yeast via yeast breaking and amplified in *E. coli*. Two colonies were picked from each of these 14 samples, and restriction digestion was carried out to investigate the sizes of the inserts. Only six of the clones contained an insert, and these are candidate numbers 4.2, 7.1, 9.1, 12.1, 13.2 and 14.1. Next, these six N_{ub} fusion plasmids were re-introduced into the NLY2 yeast strain containing the hSkp1-C_{ub}-RUra3 plasmid to test for plasmid linkage. The strength of the interaction between the two fusion proteins was quantified using ten-fold serial dilutions of cells coexpressing the fusion proteins. The serial dilutions were dropped onto WL, UWL, 100 UWL, FWL and 100 FWL plates. Figure 18 shows the growth of the cells on the various plates, and interaction between the N_{ub} fusion proteins and hSkp1-C_{ub}-RUra3 was observed.

Although the growth of cells can be seen on the plates after titration, there was a need to compare the growth of these cells relative to the negative controls to eliminate any background signals on each plate. Tim-C_{ub}-RUra3 was used as a negative control as it was localized to the cytoplasm rather than the nucleus, and the interaction of the N_{ub} fusion proteins with it is shown in Figure 18. This allowed one to see if the interaction observed between the N_{ub} fusion proteins and hSkp1-C_{ub}-RUra3 was specifically due to hSkp1 and not the C_{ub} portion. The N_{ub} expression vector pACNX-N_{ub}IBC1 was used as a negative control when testing interactions with hSkp1-C_{ub}-RUra3 transformed

Interactions of hSkp1-C_{ub}-Rura3 with the N_{ub} fusion proteins

hSkp1-C_{ub}-Rura3

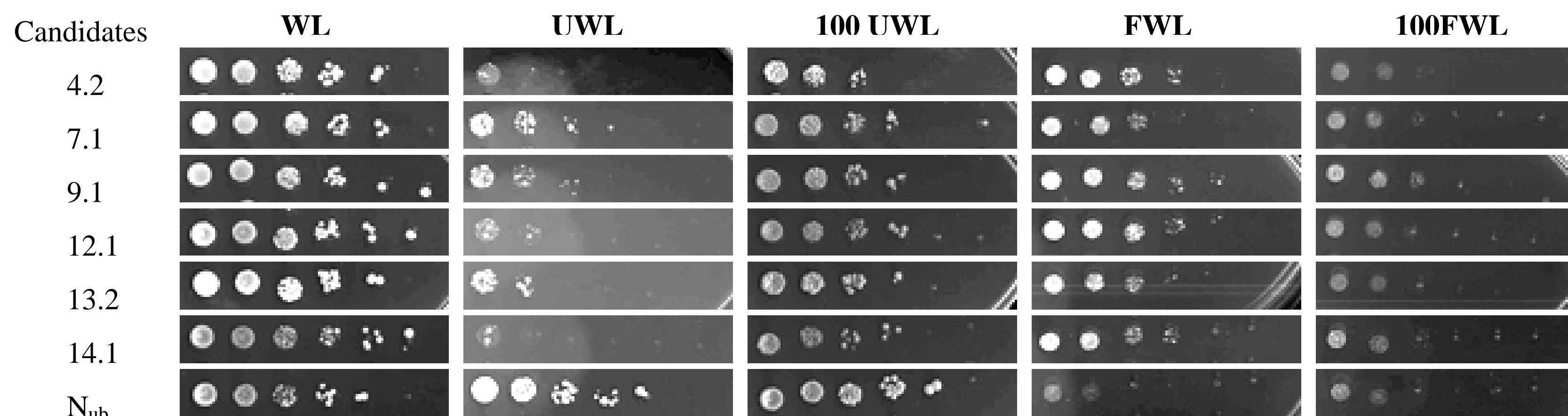

Figure 18: Ten-fold serial dilutions of cells co-expressing hSkp1-C_{ub}-RUra3 and the various N_{ub} fusion candidates were spotted onto the various plates to compare the strength of N_{ub} and C_{ub} fusion protein interactions.
Serial dilutions were in decreasing order from left (10^0) to right (10^{-5}). The lack of growth on uracil deficient plates and the ability to grow on plates containing 5-FOA indicated protein-protein interaction. N_{ub} empty plasmid was used as a negative control.

WL: plates lacking tryptophan and leucine; **UWL**: plates lacking uracil, tryptophan and leucine; **100UWL**: plates containing 100 µM $CuSO_4$, and lacking uracil, tryptophan and leucine; **FWL**: plates containing 5-FOA and lacking tryptophan and leucine; **100 FWL**: plates containing 100 µM $CuSO_4$, 5-FOA and lacking tryptophan and leucine

Interaction of Tim-C_{ub}-Rura3 with N_{ub} fusion proteins

Tim-C_{ub}-Rura3

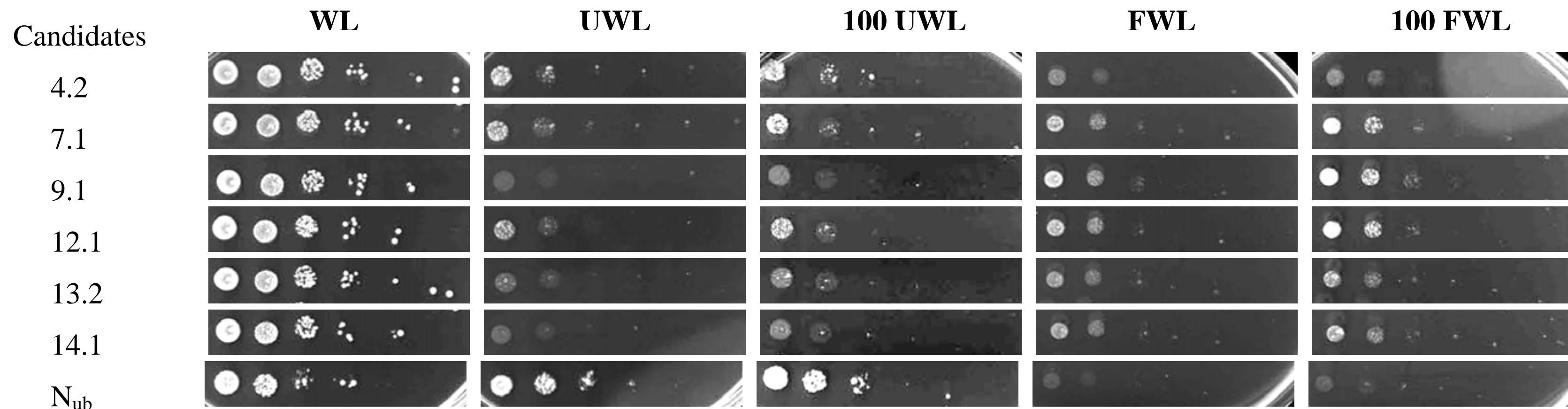

Figure 19: Ten-fold serial dilutions of cells coexpressing Tim-C_{ub}-RUra3 and the various N_{ub} fusion candidates were spotted onto the various plates to compare the strength of N_{ub} and C_{ub} fusion protein interactions.
Serial dilutions were in decreasing order from left (10^0) to right (10^{-5}). The lack of growth on uracil deficient plates and the ability to grow on plates containing 5-FOA indicated protein-protein interaction. N_{ub} empty plasmid was used as a negative control.

WL: plates lacking tryptophan and leucine; **UWL**: plates lacking uracil, tryptophan and leucine; **100 UWL**: plates containing 100 µM $CuSO_4$, and lacking uracil, tryptophan and leucine; **FWL**: plates containing 5-FOA and lacking tryptophan and leucine; **100 FWL**: plates containing 100 µM $CuSO_4$, 5-FOA and lacking tryptophan and leucine.

cells. This was to check for any background 5-FOA resistance that was not due to C_{ub} and N_{ub} fusion protein interaction.

The interaction between hSkp1-C_{ub}-RUra3 and the six N_{ub}-fusion plasmids, and Tim-C_{ub}-RUra3 and the six N_{ub}-fusion plasmids were quantified to allow comparisons with the negative controls and eliminate any background readings. Each of the double transformants was assigned a value from zero to six based on substantial growth of their serial diluted cells. For instance, when growth was seen for all six serial dilutions, this was designated a score of six. Conversely, if no growth was observed for all six serial dilutions, the value assigned would be zero. The values obtained here are termed the raw scores. Subsequently, these values were converted to relative scores when the background signals were taken into consideration. Formulae to calculate were as follows:

[(Score of double transformants on WL) – (Score of double transformant on UWL)] –

[(Score of N_{ub} on WL) – (Score of N_{ub} on UWL)]

Using Tim-C_{ub}-RUra3 / candidate 4.2 co-transformant as an example, the relative interaction score on the UWL plate was: (5-2) – (4-3) = 2.

As for the FWL plates, the formula used was as stated below:

[(Score of double transformants on FWL) – (Score of double transformant on WL)] –

[(Score of N_{ub} on FWL) – (Score of N_{ub} on WL)]

For instance, the relative score of Tim-C_{ub}-RUra3 / candidate 4.2 co-transformant on the FWL plate was: (1-5) – (0-4) = 0.

With the relative scores, an average score of the various N_{ub} fusion proteins interacting with hSkp1-C_{ub}-RUra3 was calculated; this score was obtained by taking an average of the relative scores of the four plates:

Average score = (Relative score of UWL + Relative score of 100UWL + Relative score of FWL + Relative score of 100 FWL) / 4

In order to eliminate any non-specific background growth with unrelated proteins, the average relative score for each double transformant was obtained by subtracting the average score of Tim-C_{ub}-RUra3 with the corresponding N_{ub} fusion protein. Using hSkp1-C_{ub}-RUra3 / candidate 4.2 as an example, the average relative score was obtained by the following formula:

hSkp1-C_{ub}-RURa3 / candidate 4.2 Final score = (hSkp1-C_{ub}-RUra3 / candidate 4.2 Average score) – (Tim-C_{ub}-RUra3 / candidate 4.2 Average score)

Thus the final score for hSkp1-C_{ub}-RURa3 / candidate 4.2 was (2.75 – 0.75) = 2. The interaction scores of the split-ubiquitin assay between Tim-C_{ub}-RUra3 and hSkp1-C_{ub}-RUra3 and the various N_{ub} plasmids with inserts are listed in Table 8a and b respectively. These six N_{ub} fusion plasmids were sequenced and subjected for BLAST search to identify the genes. The identities of genes from the cDNA library screen were listed in Table 9.

The expected protein sizes of each of the N_{ub} fusion proteins were also calculated (Refer to Table 9), and these plasmids were transformed into the NLY2 yeast strain to test for the expression of the fusion proteins. The Western blot was performed using an

antibody against the haemagglutinin (HA) tag present between the N_{ub} and the protein. Figure 20 shows the results of the Western blot analysis.

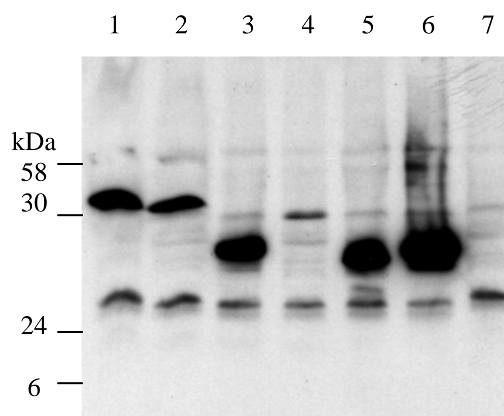

Figure 20: Expression of N_{ub} fusion proteins isolated from the library screen.
Yeast cells were transformed with the various plasmids and harvested from media deficient in leucine for the SDS-PAGE. Western Blot analysis was performed using haemagglutinin primary antibody. Lane 1 and 2: expression of candidate 4.2 and 12.1 (N_{ub}-Cdk2-interacting protein; 32kDa), Lane 3: expression of candidate 7.1 (N_{ub}-putative translation initiation factor (SUI1); 21kDa), Lane 4: expression of candidate 9.1 (N_{ub}-hypothetical protein FLJ14346; 22kDa), Lane 5: candidate 13.2 (N_{ub}-S100A6; 19kDa), Lane 6: 14.1 (N_{ub}-S100A11; 21kDa) and Lane 7: expression of N_{ub} (negative control).

Candidate	Tim-C_{ub}-RUra3 Raw score					Tim-C_{ub}-RUra3 Relative score				Tim-C_{ub} Average scores
	WL	U	100 U	FWL	100 FWL	U	100 U	FWL	100 FWL	
4.2	5	2	3	1	1	2	1	0	0	0.75
7.1	5	2	2	2	2	2	2	1	1	1.5
9.1	5	0	0	2	2	4	4	1	1	2.5
12.1	5	1	2	2	2	3	2	1	1	1.75
13.2	5	1	1	2	2	3	3	1	1	2
14.1	4	1	1	2	2	2	2	2	2	2
Nub	4	3	3	0	0	-	-	-	-	-

Table 8(a): Strength of interactions between Tim-C_{ub}-RUra3 and the N_{ub} fusion candidates

Candidate	hSkp-C_{ub}-RUra3 Raw score					hSkp-C_{ub}-RUra3 Relative score				hSkp1-C_{ub} Average scores	Final score
	WL	U	100 U	FWL	100 FWL	U	100 U	FWL	100 FWL		
4.2	5	1	3	4	2	3	2	4	2	2.75	2
7.1	5	3	4	3	2	1	1	3	2	1.75	0.25
9.1	5	2	4	4	3	2	1	4	3	2.5	0
12.1	5	1	4	4	2	3	1	4	2	2.5	0.75
13.2	5	2	4	3	1	2	1	3	1	1.75	0
14.1	5	1	3	3	1	3	2	3	1	2.25	0.25
Nub	5	4	5	0	0	-	-	-	-	-	-

Table 8(b): Strength of interactions between hSkp1-C_{ub}-RUra3 and the N_{ub} fusion candidates

Table 8: Growth scores of the interactions between the various C_{ub} fusions and the N_{ub} fusion candidates.

Raw scores (ranging from 0 to 6) were assigned by counting the number of serial dilutions where growth was observed. The C_{ub} fusion protein average score was obtained by taking the average of the calculated scores, and the final score was calculated by taking the respective C_{ub} fusion protein average score subtracting the Tim-C_{ub}-RUra3 average score.

WL: plates lacking tryptophan and leucine

U: plates lacking uracil, tryptophan and leucine

100U: plates containing 100µM $CuSO_4$, and lacking uracil, tryptophan and leucine

FWL: plates containing 5-FOA and lacking tryptophan and leucine

100FWL: plates containing 100µM $CuSO_4$, 5-FOA and lacking tryptophan and leucine

Candidate	Final score for plasmid linkage	DNA		Protein		
		Length of insert (bp)	Results of Blast from sequence using 5’ primer	Cloned in frame?	Is the ORF intact?	Western Blot (HA tagged)
4.2	2	1500	*Homo sapien*s cyclin-dependent kinase 2-interacting protein (CINP)	Yes, frame 2	1st Met missing. The 3’ untranslated region is different between the two clones.	Yes. 31 kDa as expected.
12.1	0.25	1500		Yes, frame 2		
7.1	0	650	*Homo sapiens* putative translation initiation factor (SUI1), mRNA	Yes, frame 2	Has some 5’ untranslated region. ORF is intact.	Yes. 21 kDa as expected.
9.1	0.75	850	*Homo sapiens* hypothetical protein FLJ14346, mRNA	Yes, frame 1	First 12 amino acids missing, replaced by 3 repeats of HASA.	Yes. 22 kDa as expected.
13.2	0	850	*Homo sapiens* S100 calcium binding protein A6 (calcyclin), mRNA	Yes; frame 1	Has some 5’ untranslated region. ORF is intact.	Yes. 19kDa as expected.
14.1	0.25	500	*Homo sapiens* S100 calcium binding protein A11 (calgizzarin) (S100A11), mRNA	Yes, frame 3	Has some 5’ untranslated region. ORF is intact.	Yes. 21 kDa as expected.

Table 9: Identification of genes of the N_{ub} fusion inserts sequences from the library screen.
The description of the various candidates isolated from the library screen that had an insert. The length of insert was observed from the restriction digestion with enzymes *Hind* III and *Not* I, and includes N_{ub}. Final score referred to the interaction strength of the protein pair, and nucleotide BLAST search was performed to identify the insert. Western Blot was also performed to verify the sizes of the N_{ub} fusion proteins. The frame indicated refers to the reading frame that the insert was cloned downstream to N_{ub}. ORF: open reading frame; HA: haemagglutinin tag sequence.

CHAPTER 5

DISCUSSION

5. Discussion

5.1 Interaction between hSrb7p and hSkp1p observed in yeast and human cells

According to the Morley (2003), a C-terminal portion of hSkp1p was isolated in a yeast split-ubiquitin system using hSrb7p as bait. In this first part of my thesis, I have verified that full-length hSrb7p and full-length hSkp1p indeed interact in both the yeast and mammalian split-ubiquitin system.

5.1.1 Molecular cloning

The generation of the open reading frame (ORF) of hSKP1 and hSRB7 via PCR was successful (Figure 10a), and by designing the PCR primers such that they contain the appropriate restriction sites at each end, cloning into the various vectors was made possible. The ORFs were subjected to restriction digestion and then subjected to column purification to remove the enzymes. For the yeast vectors, the ORF of hSkp1 was cloned into pACNX-N_{ub} vectors, resulting in the fusion protein N_{ub}-hSkp1, whereas for hSrb7, it was cloned into Pcup-C_{ub}-RUra3 and therefore giving rise to fusion protein Srb7-C_{ub}-RUra3. The ORF of hSkp1 was cloned into mammalian vector pcDNA3-N_{ub}I2, resulting in the fusion protein N_{ub}I2-hSkp1; as for hSrb7, it was cloned into pcDNA3-C_{ub}-RGpt2 vector thus ensuing that the final protein was hSrb7-C_{ub}-RGpt2. DNA sequencing was performed to ensure that these ORF were cloned in the right reading frame with respect to N_{ub} and C_{ub} in order to obtain fusion proteins. It was also verified that there were no missense or nonsense mutations present in the clones. The cloning of plasmid pACNX-N_{ub}I2-hSgt1 was successful as well.

5.1.2 Interaction of hSrb7-C_{ub}-RUra3 and N_{ub}-hSkp1 in *S. cerevisiae* using the yeast split-ubiquitin system

hSrb7 was cloned into yeast vector upstream of the C_{ub}. Following the C_{ub} is the uracil gene whereby the first amino acid has been replaced by an arginine. Thus for the fusion C_{ub}-RUra3, uracil prototrophy was an indication that the plasmid was successfully transformed into the yeast, and it was expressed and functional. This plasmid also contains a *TRP1* gene, therefore giving rise to tryptophan prototrophy when transformed into yeast cells. As such, yeast cells transformed with the C_{ub} plasmid were plated onto plates lacking tryptophan (W^-, designated W). pACNX-N_{ub}-hSkp1 was then transformed into the above yeast strain. The N_{ub} plasmid contains the *LEU2* gene which confers leucine prototrophy to transformed yeast cells; both plasmids are selected for by plating the transformants on plates lacking tryptophan and leucine (W^-L^-, termed WL). Subsequently, the strength of the interaction between the two fusion proteins was quantified using ten-fold serial dilutions of cells co-expressing the fusion proteins. The serial dilutions were dropped onto five different media which would be discussed in detail below.

In the yeast split-ubiquitin system, protein interactions within the cell would result in increased local concentrations of the two halves of ubiquitin, and the formation of a native-like ubiquitin moiety. The RUra3p reporter would then be cleaved off by the ubiquitin-specific proteases (UBPs), and subsequently be degraded by enzymes of the N-end rule of protein degradation. Therefore, these yeast cells would display an inability to grow on plates lacking uracil. Instead of using plates lacking just uracil, selection plates lacking uracil, tryptophan and leucine ($U^-W^-L^-$, designated UWL) were used to ensure that both plasmids were maintained in the yeast cells. As the plasmid containing the C_{ub}-

RUra3 gene was driven by the CUP1 promoter, increased expression of the fusion protein occurs when copper sulphate ($CuSO_4$) was added. Therefore, yeast cells were also plated onto the UWL selection plates with 100μM $CuSO_4$ (100UWL). In addition, the cells were also titrated onto plates containing 5-fluoro-ororatic acid, a drug counterselecting Ura3. The ability of yeast cells to grow on selection plates lacking tryptophan and leucine with the addition of 5-fluoroortic acid (designated FWL) and such plates with the addition of 100μM $CuSO_4$ (100FWL) indicated that the Ura protein had been degraded as a result of the protein-protein interaction, and therefore 5-FOA was no longer toxic to the cells.

In Figure 11, it was observed that cells co-expressing hSrb7-C_{ub}-RUra3 and N_{ub}-hSkp1 showed little or no growth on UWL and 100UWL plates, an indication that the Ura3 enzyme was not present despite the selection for the C_{ub} plasmid. On the contrary, the cells co-expressing hSrb7-C_{ub}-RUra3 and N_{ub} had abundant growth on the UWL and 100UWL plates, suggesting that uracil prototrophy was still observed. As for the FWL and 100FWL plates, profuse growth was observed for cells co-expressing hSrb7-C_{ub}-RUra3 and N_{ub}-hSkp1, but much less growth was observed for cells co-expressing hSrb7-C_{ub}-RUra3 and N_{ub} empty vector. Taken together, these observations indicated that hSrb7-C_{ub}-RUra3 and N_{ub}-hSkp1 interacted in the yeast cells, and accordingly the native-like ubiquitin molecule was formed. As a result, the RUra3 reporter was cleaved by the Ubps, and subsequently degraded by the enzymes of the N-end rule. Therefore these cells were not able to grow on media lacking uracil, but survived in media containing 5-FOA, since it was no longer toxic to the cells. As a positive control, cells were also plated onto plates lacking tryptophan and leucine (WL), and as expected, all the transformants grew well on this plate. In conclusion, the interaction of hSrb7-C_{ub}-RUra3 and N_{ub}-hSkp1 was

observed in yeast cells using the yeast split-ubiquitin system, which indicated that both full-length proteins could interact.

5.1.3 Construction of stable HT1080HPRT$^-$::hSrb7-C_{ub}-RGpt2 cell line

Now that the interaction between hSrb7p and hSkp1p has been observed in yeast cells, I went on to investigate if the interaction between this pair of proteins would also be observed in human cells using the mammalian split-ubiquitin system (Rojo-Niersbach *et al*., 2000). It is important to verify that the protein-protein interaction is observed in mammalian cells because these two proteins should interact in their native environment.

HPRT enzyme activity is required for the phosphoribosylation of hypoxanthine and guanine, salvaging them for the biosynthesis of nucleic acids (Albertini, 2001). The enzyme Gpt2p from *Escherichia coli* is able to complement the deficiency of HT1080HPRT$^-$ cells. Thus the HT1080HPRT$^-$ cells that are stably transfected with the C_{ub} vectors are expected to be resistant in medium containing HAT (HAT^R), and sensitive to medium containing 6-TG ($6TG^S$). The sensitivity to medium containing 6-TG is due to the fact that the Gpt2p enzyme is able to phosphoribosylate the purine analogue 6-TG, and this step causes its cytotoxicity to the cells. On the other hand, these cells can survive in HAT medium as the GPT2 gene and the presence of hypoxanthine (a purine) in the medium allows for a functional salvage pathway for nucleotide synthesis.

hSrb7-C_{ub}-RGpt2 was observed to be stably integrated into two HT1080HPRT$^-$ cell lines as seen by the phenotype of the cell line – resistance in medium containing HAT and sensitive to medium containing 6-TG and zeocin. The addition of zeocin which selected for the plasmid was important. This was because medium containing 6-TG

selected against the C_{ub} vectors, and cells grown in this medium might have lost the plasmid in order to survive. As zeocin exerted a selection pressure on the cells to retain the plasmids, any cell line that is resistant in HAT, but sensitive in 6-TG with zeocin medium, is the ideal cell line that is expressing the Gpt2p. One of the key difficulties faced in the construction of a stable hSrb7-C_{ub}-RGpt2 cell line was the isolation of single colonies after transfection. This step was critical as subsequent steps depended on the phenotype of the cells in various media to detect if there were any protein interactions. Different cell lines could have differently integrated plasmids, and picking more than a single colony would result in inconsistencies in the phenotype detected, giving rise to inaccurate results in later stages of the experiment.

5.1.4 Interaction of hSrb7-C_{ub}-RGpt2 and N_{ub}-hSkp1 in HT1080HPRT$^-$ cells using the mammalian split-ubiquitin system

The mammalian split-ubiquitin system differs from the yeast system in that the reporter protein used is the RGpt2 protein, where the first amino acid has been modified to an arginine. Should the protein fused to C_{ub}-RGpt2 interact with the protein fused to N_{ub}, a native-like ubiquitin would be reconstituted when the two halves of ubiquitin are brought into close proximity. This would result in the cleavage of the reporter protein RGpt2, and its degradation by the enzymes of the N-end rule. Thus the HT1080HPRT$^-$ cells co-expressing the two proteins that interact would revert to being deficient in the ability to salvage nucleotides. These cells would be sensitive to HAT but resistant to 6-TG. For the construction of stable N_{ub} cell lines, G418 had been added to the medium containing HAT. This was because HAT selected for the C_{ub} plasmid while G418 selects for the N_{ub} plasmid, ensuring that neither plasmid is lost. As for medium containing 6-

TG, zeocin has been added as mentioned above. The co-expression of N_{ub} alone acted as a negative control.

One of the hSrb7-C_{ub}-RGpt2 cell lines was used for the transfection of N_{ub}-hSkp1. Selection in the various media was carried out, and the observed phenotype is shown in Figure 12. It was observed that the cells co-expressing hSrb7-C_{ub}-RGpt2 and just the N_{ub} alone remained HAT^R and $6TG^S$, and this was as expected since N_{ub} by itself does not interact with C_{ub}. As for cells co-expressing hSrb7-C_{ub}-RGpt2 and N_{ub}-hSkp1, the phenotype was HAT^S and $6TG^R$; this indicated that interaction between hSkp1p and hSrb7p was observed. As these two proteins were brought into close proximities, a native-like ubiquitin moiety was reconstituted, and thereby resulting in the cleavage of reporter protein RGpt2 and its degradation via the N-end rule. The degradation of Gpt2p caused the cells to now revert to HAT^S and $6TG^R$. To summarize this part of the discussion, the interaction of full-length hSrb7p and full-length hSkp1p was observed in human cells using the mammalian split-ubiquitin system.

Now that protein-protein interactions between the two proteins have been established, the next step was to understand the functions of hSkp1p with respect to its interaction with hSrb7p. The latter is a subunit in both the yeast and mammalian Mediator complex, and ySrb7p has been shown by Gromoller and Lehming (2000) to interact with the repressor Tup1p. As for hSkp1p, one of the best defined roles of this protein is as an adapter in the Skp1-Cullin-F-box (SCF) E3 ubiquitin ligase complex. It acts as a linker between the Cullin protein Cul1p and RING finger protein Hrt1p, and the F-box proteins as shown in Figure 4 (Zheng *et al.*, 2002). The SCF ubiquitin ligase complex acts together with the E1 and E2 enzymes in the process of poly-ubiquitylation

of target proteins, resulting in their degradation via the 26S proteasome. However, ySkp1p also has roles either than as an adapter in the SCF ligases complex, for instance, ySkp1p has been shown to be involved in endocyctosis (Galan *et al*., 2001) and the formation of kinetochore complex (Kaplan *et al.,* 1997). Most of the proteins that interact with Skp1p contain an F-box motif which binds to the C-terminal helical extension of Skp1p, and examples of such proteins include Grr1p in yeast and Skp2p in humans. But ySkp1 also interacts with proteins that lack an F-box motif, like Cul1p, Sgt1p, Rav1p and Rav2p; another indication that the role of Skp1p is not restricted to the SCF complex. As for Srb7p, it is not known to contain an F-box sequence motif.

Another intriguing point is that Skp1 has some homology to a transcriptional elongation factor (TCEB1), and was therefore termed TCEB1-L (Sowden *et al.* 1995). Incidentally, TCEB1 encodes for ElonginC, a eukaryotic transcription factor that is part of the SIII elongation complex. As discussed in Section 2.1.2 and 2.2.2, ElonginBC can assemble with Cul2p, Hrt1p and Med8p to reconstitute an ubiquitin ligase. Med8p is a component of the Mediator complex and possesses a conserved BC-box motif to which ElonginC binds. My work now suggests that another subunit of the Mediator complex, hSrb7p, interacts with hSkp1p which is a component of the SCF E3 ubiquitin ligase. Although Schulman *et al*. (2000) have shown that hSkp1p binds the F-box protein hSkp2p differently from the way ElonginC binds to BC-box protein von Hippel-Lindau (VHL), the arrangement of the helixes in the two interfaces are similar. However, one clear difference is the location of hMed8p and hSrb7p in the Mediator complex: hMed8 is located in the head domain of the Mediator complex whereas hSrb7p has been shown to localize to the Middle domain (Figure 1). Nonetheless, this could imply that hMed8p and

hSrb7p could recruit ubiquitin ligase activity to the Mediator or the holoenzyme, possibly leading to the poly-ubiquitylation of the target and its degradation by the 26S proteasome. The Mediator complex is already known to possess histone acetyl-transferase activity HAT through subunit Nut1p (Lorch *et al*., 2000). However, as NUT1 is a non-essential gene in yeast, HAT activity was not believed to be a major role of the Mediator complex. In this case, since SRB7 is an essential gene in yeast, this may imply that the E3 ubiquitin ligase activity may, indeed, play an important role for the Mediator complex.

5.2 Skp1p is required for maximal induction of Hsp70B' RNA

5.2.1 The rationale behind the experiment

As discussed earlier, the interaction of hSrb7p and hSkp1p may bring the E3 ubiquitin ligase activity to the Mediator complex. Alternatively, hSkp1p could act as a transcription factor since it is homologous to a transcriptional elongation factor. Thus I wanted to investigate the effects of hSkp1p on transcription. This part of the experiment encompasses finding an inducible transcription promoter and studying its transcript levels when hSkp1 mRNA levels are reduced using RNA interference.

Short-interfering hSkp1 RNA targeting the 3' untranslated region (3' UTR) of hSkp1 mRNA was produced using the pSuper RNAi system. The vector pSuper uses the polymerase III-H1 RNA gene promoter to produce a small RNA transcript which lacks a polyadenosine tail, and the transcript also has a well-defined transcription start site and a termination signal made up of 5 thymidines. It is predicted to fold back on itself to form a 19-nucleotide pair stem-loop structure, and it is believed that such stem-loop precursor transcripts are then quickly cleaved in the cell to produce a functional siRNA. The human

Heat Shock protein 70 (HSP70) promoter was chosen as it is under selective and inducible transcriptional regulation that is well-studied in eukaryotes. Cells respond to temperatures above their optimum for growth by producing these heat shock proteins (with 70,000 kDa being the most prominent), and the response is rapid; within minutes of heat induction, transcription is initiated at the *hsp* gene loci. There are 4 known heat-inducible Hsp70 genes in humans (Parisan *et al.,* 2000), and the Hsp70B' was selected because according to Parisan *et al*. (2000), the transcripts of this gene was strongly induced upon heat-induction. Because of its efficiency of induction of transcription, the heat-shock promoters, especially the hsp70 promoters, have been used for gene therapy strategies (Rome *et al*., 2005).

Two-step reverse transcription polymerase chain reaction (RT-PCR) using the SYBR-Green dye was employed to study the amounts of hSkp1 and Hsp70B' RNA. Untreated HeLa cells referred to cells that were treated with empty vector pSuper, and not hSkp1 siRNA. On the other hand, treated cells referred to those transfected with hSkp1 siRNA. Forty-eight hours after transfection, the cells were then either subjected to heat-induction or non-heat shocked (NHS). Heat treatment referred to cells being placed at 42°C for 15 minutes; these cells then had a recovery period of an hour at 37°C. Subsequently, total RNA was harvest from all samples, and the transcripts of hSkp1 and Hsp70B' were analyzed by reverse transcription and quantitative real-time PCR by SYBR-Green dye. The endogenous control used was the human glyceraldehyde-3-phosphate dehydrogenase (GAPD).

Real-time PCR assays were used in this part of the experiment as I wanted to track the effects of reduction of hSkp1 on the activated transcription of Hsp70B'. This

assay is highly sensitive to difference in amount of starting sample, as compared to PCR where only the end products are analyzed. The SYBR-Green dye chemistry was used in the assay, and the dye binds to double-stranded DNA products formed during PCR. It was chosen instead of the TaqMan chemistry as the SYBR-Green could be used to monitor both the hSkp1 and Hsp70B' RNA levels (in separate reactions). In addition, it does not require additional probes, therefore reducing costs. However, false-positive results may arise as SYBR-Green dye binds unspecifically to any double-stranded DNA sequences. Thus a dissociation (melting) curve was carried out at the end of the real-time PCR to ensure that there was no non-specific products formed during the run. At the melting temperature of the amplicon, only a single sharp peak was observed, and the non-template control did not yield any fluorescence. In addition, the real time PCR was performed in duplicates to control pipetting errors. A non-template control where all the PCR reagents were added except the RNA template was included as a negative control; as expected, no fluorescence was detected for this sample. At the end of the run, relative quantitation by the comparative C_T method was then used to analyze the changes in RNA levels of hSkp1 and Hsp70B' relative to the sample that was not subjected to transfection with hSkp1 siRNA and non-heat induced (Figure 15, 'untreated, NHS').

5.2.2 Discussion of the results

The cloning of the pSuper-681-hSkp1 plasmid was successful (Figure 13), and transfection of this plasmid into HeLa cells resulted in some reduction of hSkp1 protein levels (Figure 14). In the second part of the experiment, RT-PCR was performed for the various samples. Relative quantitation of the data using the comparative C_T method was

carried out, and calculations are shown in Table 6 for analysis of hSkp1 mRNA and Table 7 for Hsp70B' mRNA. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence can be detected; thus a smaller threshold cycle (C_T) value indicates that fewer cycles are needed for the fluorescence to reach a fixed threshold level.

From Figure 15a, it was seen that the transfection of cells with hSkp1 siRNA had resulted in two-fold decrease in hSkp1 mRNA compared to untreated cells. Induction of cells with heat did not affect the hSkp1 transcripts in both untreated and treated cells. RNA interference using hSkp1 siRNA did not result in the elimination of hSkp1 transcripts. One explanation for this could be that Skp1 is an essential gene in *S. cerevisiae* (Giaever *et al.,* 2002), and it may be also be an gene required for cell viability in human cells. Therefore, the levels of Skp1 mRNA were not eliminated since the human cells would likely be dead if this protein was absent.

Next, the transcript levels of inducible Hsp70B' gene was investigated. It was observed that for the untreated cells, upon heat-shock at 45°C for 15 minutes, there was a significant increase in the Hsp70B' mRNA levels as compared to the non-heat shocked (NHS) cells (Refer to the data in Table 7 and Figure 15b). The 217-nucleotide amplicon increased 268-fold in the heat-shocked sample compared to NHS cells. This was as expected as many articles have reported that cells respond within minutes of rise in temperature to produce heat-shock proteins. The transfection of hSkp1 siRNA in NHS cells did not have an effect on the Hsp70B' basal level. This was an important control to show that the hSkp1 siRNA does not affect the Hsp70B' transcripts. However, when the cells were treated with hSkp1 siRNA and induced with heat, there was only a 60-fold

increase in Hsp70B' transcripts as compared to the NHS hSkp1 siRNA-treated sample. In a comparison between the two heat-shocked samples, the sample that was treated with hSkp1 siRNA resulted in a 4-fold decrease in Hsp70B' transcript versus the sample that was untreated. In conclusion, the reduction of hSkp1 had reduced the transcripts levels of induced Hsp70B' gene, indicating that hSkp1p is required for the maximal induction of Hsp70B'.

From these data, we can say that hSkp1p has a positive effect on induction of the Hsp70B' RNA. Its interaction with hSrb7p could affect the holoenzyme in such a way that transcription initiated is activated. There are several possibilities as to how hSkp1p may act to increase the transcription efficiency of Hsp70B'. Given the increased evidence of the cross-talk between ubiquitin-proteasome pathway and transcription regulation, it is an attractive idea to postulate that the recruitment of hSkp1p to the promoter by hSrb7p would result in the attraction of the SCF E3 ubiquitin ligase, resulting in the poly-ubiquitylation of transcription factors like repressors. With the degradation of the repressor, efficient transcription of Hsp70B' mRNA would occur. This simplified model would imply that hSkp1 works at the transcription initiation level, and involves the SCF ligase.

Intriguingly, there is a possibility that hSkp1p may act at the transcription elongation stage and not the transcription initiation phase. As mentioned earlier, hSkp1p is homologous to Elongin C. The latter was first discovered together with Elongin B and Elongin A as part of the Elongin complex (SIII), and this complex was shown to stimulate the rate of elongation of RNAPII in mRNA synthesis (Bradsher *et al*., 1993a, b). However, the exact mechanisms of how the SIII complex affects elongation have not

been deduced up to this day. In this proposed model, hSkp1p would work independently of the SCF E3 ubiquitin ligase. It would be recruited to the holoenzyme by Srb7p, resulting in the enhancement of the transcription elongation ability of the RNAPII. This theory is similar to the work by Gerber *et al.* (2005). The expression of heat shock genes was believed to be regulated at the promoter clearance phase, and therefore the transcription of such genes are strongly affected by the recruitment of RNAPII elongation factors to the promoter. It was shown that in *Drosophila melanogaster*, dEloA, an Elongin A homologue, was involved in the regulation of heat shock gene. Should hSkp1p function as a transcription elongation factor, this could explain why a decrease in Hsp70B' mRNA was detected when Skp1 transcripts decreased.

Another possible scenario is that hSkp1p may attract the SCF ligase complex, resulting in the ubiquitylation of a factor involved in transcription. This protein could range from a Mediator subunit, histones, RNA polymerase II or activators and repressors at the promoter region (Muratani and Tansey, 2003). This would be in accordance with the work by theory that the Elongin BC complex, together with BC-box containing Elongin A, was speculated to be responsible for the ubiquitylation of RNAPII or other transcriptional factors (Shilatifard *et al.*, 2003). We now know that the large subunit of RNAPII becomes ubiquitylated when exposed to DNA damaging agents like UV, and the function of the ubiquitylation of RNAPII was to degrade it and allow the DNA repair machinery to be recruited to the site. However, the exact function of hSkp1p has not been elucidated, and more work remains to be carried out.

5.3 Cloning of complementary DNA (cDNA) library fused to N_{ub}

The isolation of total RNA was successful as shown in the integrity and sizes of the ribosomal bands on the denaturing formaldehyde gel (Figure 16). Both the 18S and 26S ribosomal RNA appeared as sharp bands when viewed under UV and this indicated that the RNA sample did not suffer any major degradation during the isolation. mRNA was isolated from the total RNA, and subsequently, the first strand and second strand synthesis of cDNA was performed. The resulting cDNA was constructed such that they had *Sal* I-*Not*I restriction sites generated at the 5' and 3' end for directional cloning into the N_{ub} plasmids. After the ligation of cDNA to the vectors and transformation into *E. coli* cells, twenty colonies were picked for analysis of cloning efficiency. Of twenty plasmids, six were observed to contain an insert. Given that that total number of clones was estimated to be 4 x 10^5, this means that 30% of them carried an insert. Therefore I estimate that about 1.2 x 10^5 clones contain an insert, and only a third of these are expected to be in frame with N_{ub} (4 x 10^4) to give rise to fusions with real proteins.

To create a library, one can choose to construct a genomic DNA (gDNA) library or complementary DNA (cDNA) library. The use of gDNA library may not be suitable for protein-protein interaction assays since it may contain non-coding sequences (introns) which have to be removed before the protein can be functional and fold into the right conformation. Thus, the library was constructed using cDNA isolated from HeLa cells. The Superscript™ plasmid system (Invitrogen) was chosen because it was able to produce a directional library instead of random library. This means that all the members in the cDNA library contain cDNA inserts cloned in a specific orientation relative to the transcriptional polarity of the original mRNAs; members of a random library will have

inserts in either orientation. As such, all members of the directional library have the potential the express a functional mRNA. However, as this library produces fusion proteins where the cDNA are cloned downstream of the N_{ub}, only one-third of the members are expected to be in the right reading frame. The cDNA library should ideally contain all mRNA present in a cell to be representative. Because many mRNAs are only expressed in low levels – as little as 1 in 10^6 of total mRNA, the cloned cDNA library should contain at least 1 million clones. To improve the cloning efficiency of the cDNA library, I would propose performing column-size fractionation of the cDNA fragments before ligating them to the digested vectors. This removes inserts which are too small and may not give rise to functional proteins. This is because it has been noted that the smaller the insert fragment, the easier it is to clone into the plasmid.

5.4 N_{ub}-cDNA library screen using hSkp1-C_{ub}-RUra3 as bait in the yeast split-ubiquitin system

hSkp1 was cloned upstream of C_{ub}-RUra3, and it was important to ensure that the resultant fusion protein was functionally active. This was because there was a possibility that the protein was unable to fold into the right conformation due to steric hindrance. Yeast cells transformed with this plasmid had been shown to be able to grow on plates lacking uracil (U^-), an indication that the Ura3 enzyme was active. In addition, ySgt1, a protein known to interact with ySkp1, and therefore the human protein pairs were tested if they would interact (Kitagawa *et al*., 1999). Here, hSgt1 was cloned downstream of N_{ub}, therefore giving rise to fusion protein N_{ub}-hSgt1. It was observed in Figure 17 that cells co-expressing hSkp1-C_{ub}-RUra3 and N_{ub}-hSgt1 did not grow very well on plates lacking uracil, but grew well on media containing 5-FOA. This indicated that hSkp1 and

hSgt1 indeed interact. As hSkp1 was also documented to be able to form dimers, N_{ub}-hSkp1 was used as a positive control. Similarly, cells co-expressing hSkp1-C_{ub}-RUra3 and N_{ub}-hSgt1 exhibited poor growth on media lacking uracil, and abundant growth was observed on media containing 5-FOA. On the contrary, cells co-expressing hSkp1-C_{ub}-RUra3 and N_{ub} (negative control) grew well on plates lacking uracil and did not survive on media containing 5-FOA. As previously discussed in Section 5.1.2, the interaction of two proteins would result in the cleavage and degradation of the reporter protein RUra3 by the enzymes of the N-end rule. This means that the cells display uracil auxotrophy and resistance to 5-FOA should they co-express an interacting pair of proteins. Therefore, these phenotypes were an indication that N_{ub}-hSkp1 and N_{ub}-hSgt1 interacted with hSkp1-C_{ub}-RUra3.

Subsequently, the yeast split-ubiquitin assay was used to find interacting partners of hSkp1 by screening the library of human cDNA fused downstream of N_{ub}. The N_{ub} library constructed was transformed into a yeast strain containing hSkp1-C_{ub}-RUra314 plasmid. The transformed cells were then plated onto WL, FWL, and 100 FWL like mentioned in Section 5.1.2. The plating of cells on the WL plate was carried out in dilutions of 10^{-3}, 10^{-4} and 10^{-5}, and 50 colonies were obtained on the 10^{-5} dilution. This meant that there were 5,000,000 independent primary transformants. Protein-protein interactions within the cell would result in increased local concentrations of the two halves of ubiquitin, and the formation of a native-like ubiquitin moiety. The RUra3p reporter would then be cleaved by the ubiquitin-specific protease (UBPs), and subsequently degraded by enzymes of the N-end rule of protein degradation. As such, the ability of yeast cells to grow on the selection plates FWL and 100 FWL indicated that the

Ura3 protein had been degraded, and therefore 5-FOA was no longer toxic to the cells. Fourteen yeast colonies co-expressing the C_{ub} and N_{ub} fusion proteins grew on the selection plates.

These 14 colonies were then subjected to yeast breaking, and the plasmids recovered were amplified in *E. coli*. The plasmids were subjected to restriction digestion to find out the sizes of the inserts (Table 9). As the plasmids that were of relevance to the library screen should carry an insert, thus only candidate 4.2, 7.1, 9.1, 12.1, 13.2 and 14.1 were subjected to further tests. These six plasmids were tested for plasmid linkage to establish if they were indeed responsible for the change in phenotype observed on media lacking uracil and containing 5-FOA. The strength of the interaction between the two fusion proteins was quantified using ten-fold serial dilutions of cells co-expressing the fusion proteins. The serial dilutions were dropped onto WL, UWL, 100 UWL, FWL and 100 FWL plates. Figure 18 shows the growth of the cells on the various plates, and cells co-expressing the N_{ub}-fusion proteins and hSkp1-C_{ub}-RUra3 showed weak uracil auxotrophy, and 5-FOA resistance. This meant that the six candidates interacted with hSkp1. However, a comparison of the strength of all the different protein pairs was necessary to eliminate background signals. Therefore scores ranging from 0 to 6 were assigned to the number of ten-fold serial dilutions for which substantial growth was observed on each media. This was taken as the raw score (Table 8b). The calculation for the average score for each pair of proteins was as listed in Section 4.7. An average relative score was obtained after the removing any back-ground signal by subtracting the Tim-C_{ub} (Table 8a) and N_{ub} average score. The final average score for all the protein pairs was listed in Table 8b as the final score. Looking at the final scores obtained, it

would suggest that candidates 9.1 and 13.2 did not interact with hSkp1, whereas candidates 7.1, 14.1 and 12.1 interacted weakly with hSkp1, and a strong interaction score of 2 was obtained for candidate 4.2 and hSkp1. In addition, DNA sequencing and BLAST results revealed the identity of the six candidates as the following: 4.2: cyclin-dependent kinase 2-interacting protein (CINP); 7.1: putative translation initiation factor (SUI1); 9.1: hypothetical protein FLJ14346; 13.2: S100 calcium binding protein A6 (calcyclin) and 14.1: S100 calcium binding protein A11 (calgizzarin) (Table 9). Incidentally, candidate 12.1 was identified as cyclin-dependent kinase 2-interacting protein (CINP) identical to candidate 4.2, but the sequences in the 3'untranlsated region for both plasmids were different. This difference may have affected the stability of the construct, resulting in different protein levels (Figure 20). The differences in interaction with hSkp1-C_{ub}-RUra3 and Tim-C_{ub}-RUra3 between candidate 4.2 and 12.1 could also be attributed to this reason (Figure 17, Figure 18 and Table 8a,b).

If the interactions which had a final score of 1 and above are taken as true interacting pairs, this indicates that only the Cdk2-interacting protein (CINP) was found from the screen to interact with hSkp1. As mentioned in Section 2.2.3.1, hSkp1 is part of the SCF E3 ubiquitin ligase essential for cell-cycle transition, specifically via the cyclins and their cyclin-dependent kinases (Cdk). CINP had been isolated in a large-scaled screen and analysis of human cDNA sequences (Strausberg *et al*., 2002), and this protein was recently shown to be associated with active cyclin E/Cdk2 and cyclin A/Cdk2 complexes (Grishina and Lattes, 2005). It was proposed that CINP may be an important link between Cdk2 and cell division cycle gene (Cdc7) complexes at the origin of DNA replication. This could mean that hSkp1 may play a role in DNA replication as well. In

addition, as mentioned in Section 2.2.3.1, Skp1 is involved in cell-cycle progression where Cdk2 plays a role as well. SCF^{Skp2} ubiquitylates phosphorylated p27, leading to the activation of Cdk1 and Cdk2 at the G1-S phase transition (Sheaff *et al.,* 1997; Montagnoli *et al*., 1999; Vlach *et al*., 1997). As for SCF^{Cdc4}, it targets cyclin E for ubiquitylation; for cyclin E to be degraded, it must first be phosphorylated by Cdk2 and glycogen-synthase kinase 3β (Koepp *et al.*, 2001; Strohmaier *et al*., 2001). Therefore it is possible that CINP may play a role in cell-cycle as well since both hSkp1 and Cdk2 regulate the mitotic cell-cycle. The next step is to use a different protein-protein interaction assay like co-immunoprecipitation to prove that hSkp1 and CINP are directly in contact and not just in close proximity.

Another candidate 13.2 which was identified as the S100A6 protein had been shown to interact with hSgt1 *in vitro* (Nowotny *et al*., 2003), and ySgt1 is known to be associated with ySkp1 as discussed in Section 2.2.3.3. However, no interaction between S100A6 and hSkp1 was documented. This protein was probably isolated because it was in close proximity with hSkp1 since the split-ubiquitin system is able to isolate proteins that are in close proximity.

In the N_{ub}-cDNA library screen, no established interacting partner of hSkp1p was isolated. There are several reasons for this. As mentioned in Section 2.2.3.2, hSkp1 interacts with F-box proteins is in the C-terminus helical fold region. However, the library screen I conducted was using a bait where the C-terminal of hSkp1p was fused to C_{ub}-RUra3. The fusion of the reporter to the C-terminus of hSkp1 could result in steric hindrance, and decreased accessibility of interacting partners to bind to hSkp1p. Unfortunately, switching the fusion of hSkp1p from the N-terminus of C_{ub} to the C-

terminus of C_{ub} is not feasible. This is because the ubiquitin specific proteases (Ubps) cleave off proteins linked to the C-terminus of ubiquitin. To overcome this problem, one can try to improve on the linker sequence between the two macromolecules to allow each protein domain to be independently functional (George and Heringa, 2003). Secondary structural elements that may form helical or β-sheet structures should be avoided as they will interfere with the flexibility of the fusion proteins, and the amino acid sequences of the linker could also affect the folding stability of a fusion protein (George and Heringa, 2003). Taking these factors into consideration, I propose placing a linker sequence made up of small amino acids like glycines, alanines and prolines. Proline is unique among all the amino acids in that it cannot form hydrogen bond to any surrounding amino acids, therefore preventing the formation of any structure with surrounding domains. A proline pair is present in the current vector construct, and perhaps increasing this number may present interference between the two domains. Either then the C-terminal helical fold region, hSkp1 has also been shown to interact with Cullin 1 in the SCF complex via the N-terminus portion. Thus, there is a possibility that by screening a larger number of colonies, more clones would be covered, and therefore increasing the chances of picking up known interacting proteins. A different control could be also established for the screening of mammalian proteins using the yeast split-ubiquitin system. Currently Tim-C_{ub}-RUra314 has been used to show that the N_{ub} fusion proteins interacted specifically with hSkp1-C_{ub}-RUra3 and not the C_{ub} portion. However, Tim is a yeast protein, and perhaps the use of a mammalian protein may be better. Alternatively, green-fluorescence protein (GFP) fused to C_{ub}-RUra3 could be used as a control.

5.5 Conclusion

The interaction of full-length hSkp1p and full-length hSrb7p was observed in *S. cerevisiae* using the yeast split-ubiquitin system, and in human cells using the mammalian split-ubiquitin system. RNA interference to knock down hSkp1 mRNA was successful in reducing it by 2-fold. This reduction in hSkp1 mRNA resulted a 4-fold reduction of activated Hsp70B' transcripts, indicating that hSkp1 was required for the efficient expression of activated Hsp70B' transcripts. Therefore, these results suggested that hSkp1p could be recruited to the promoter via hSrb7p, and that it is involved in transcription. A cDNA library was cloned for the study of protein-protein interactions using the yeast-split ubiquitin system. A screen for interacting partners of hSkp1p was performed in hope of finding interacting partners that would help us in understanding the functions of hSkp1p. Cyclin-dependent kinase 2-interacting protein (CINP) was isolated as in interacting partner of hSkp1p.

5.6 Future work

The interaction of hSrb7p and hSkp1p was observed in mammalian cells, and I think it would be interesting to find out the sequences in hSrb7 that mediate the binding of these two proteins. This is because hSkp1p is known to bind to F-box proteins that as their name implied, carry an F-box sequence domain. Although it is highly unlikely that hSrb7p possesses such a protein motif, the discovery of the exact sequences to which hSkp1p binds to may allow us to identify other candidates that hSkp1p may interact with. In addition, the N-terminus of Srb7p is well conserved in species like *S. cerevisiae*, *Drosophila melanogaster* and *Homo sapiens*. Should the interaction domain be in this

region, there is a high probability that similar interacting protein pair would also be found in the organisms mentioned above.

If the interaction of hSrb7p and hSkp1p recruits the SCF ubiquitin ligase activity, which protein is the target of this complex to be poly-ubiquitylated and degraded? What role does hSkp1p play in transcriptional initiation and possibly transcription elongation? These answers to these questions remain to be discovered. I have proposed several experiments to investigate the functions of hSkp1p and hSrb7p.

In *D. melanogaster*, the Mediator complex has been shown to be rapidly recruited to the heat shock promoter upon heat shock (Park *et al*., 2001), and this includes Srb7p as well. Therefore, if we assume that hSrb7p would also be recruited to the human heat shock promoter upon heat shock, this suggests that hSkp1p could be recruited via hSrb7p. The use of chromatin immunoprecipitation (Ch-IP) assay would definitely be useful in this aspect to examine if both proteins are found at the *HSP* loci and are dependent on heat-shock stimulus. In addition, RNA interference to knock down hSrb7 mRNA could also be coupled with the Ch-IP experiment to observe if the reduction in hSrb7 has any effects on the recruitment of hSkp1p to the promoter.

In addition, the use of RNA interference to reduce the mRNA of hSrb7 and examine how this affects the Hsp70B' transcripts would help us understand the role of hSrb7p too. I would assume that similar to that of reduction of hSkp1, the reduction in hSrb7 levels will result in decreased levels of activated Hsp70B' transcripts. If this is so, it is an indication that these two proteins are required for the maximal activation of Hsp70B'. However, if the reduction in hSrb7 transcript levels results in an increase in

Hsp70B' mRNA, this means that hSrb7p acts like a repressor, and exerts a different effect from Skp1p.

Since it is speculated that hSkp1p may recruit the ubiquitin ligase activity to the holoenzyme, resulting in the poly-ubiquitylation of a factor like a repressor and its degradation via the 26S proteasome, the use of proteasomal inhibitor MG132 would be appropriate. For instance, the reduction of hSkp1p via siRNA has resulted in a decrease in activated transcripts. When MG132 is added, the proteasome pathway would be blocked and presumably the repressor would not be degraded. Therefore one would observe a similar decrease in activation of transcript levels. As such this result would indicate that hSkp1 and the proteasome work in the same pathway. However, the use of MG132 proteasome inhibitor is not suitable for the Hsp70B' gene since the addition of such inhibitors has an effect on basal transcription and causes an increase in production of heat-shocked proteins in the absence of heat-shock. Thus a different inducible system is required.

To identify the targets of the SCF ubiquitin ligase complex, I would suggest to look at transcription factors that are known to be phosphorylated. This is because phosphorylated substrate proteins like yeast Sic1p (Deshaies, 1997; Feldman *et al*., 1997 and Verma *et al*., 1997) act as recognition signals for the SCF ubiquitin ligase complex. An example would be that of the activator Gcn4p which is phosphorylated by Srb10p after it is recruited to the holoenzyme at the promoter, and this modified form of Gcn4p is subsequently recognized by SCF^{Cdc4} and degraded by the SCF complex (Irniger and Braus, 2003).

As for the protein-protein interaction screen using hSkp1-C_{ub}-RUra3 as bait, more work has to be done to further characterize the interaction between hSkp1p and the various candidate proteins isolated with the yeast split-ubiquitin system. For instance, *in vitro* assays should be carried out to verify the interactions since the split-ubiquitin assay indicates that the proteins are in close proximity, but not necessarily interacting proteins. For instance, one could use the glutathione-S-transferase (GST) fusion system to express hSkp1-GST fusion proteins and perform a GST pull-down assay. In addition, the use of RNA interference to reduce the levels of the genes of the isolated proteins and study the effects on hSkp1p would allow one to see the function of their interaction. This is especially the case for the cyclin-dependent kinase 2-interacting protein (CINP) isolated in the screen; its protein-protein interaction with Cdk2p should be tested and characterized as well.

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APPENDICES

APPENDICES

Appendix 1: Preparation of LB with amipicillin/chloramphenicol plates (1 litre)

- Components of the plates

Tryptone	10 g/L
Yeast Extract	5 g/L
Sodium chloride	5 g/L

-Add 800ml of distilled water, and 0.2ml 5N sodium hydroxide to adjust the pH.
-Mix well. Add 15 g/l Bactoagar. Autoclave, and place in 50°C water bath to cool.
-Add respective amounts of ampicillin / chloramphenicol to media and pour onto plates.

Appendix 2: Preparation of plates for culturing of yeast

i. The amino acid premix* for 20L of synthetic complex media is prepared according to the table below:

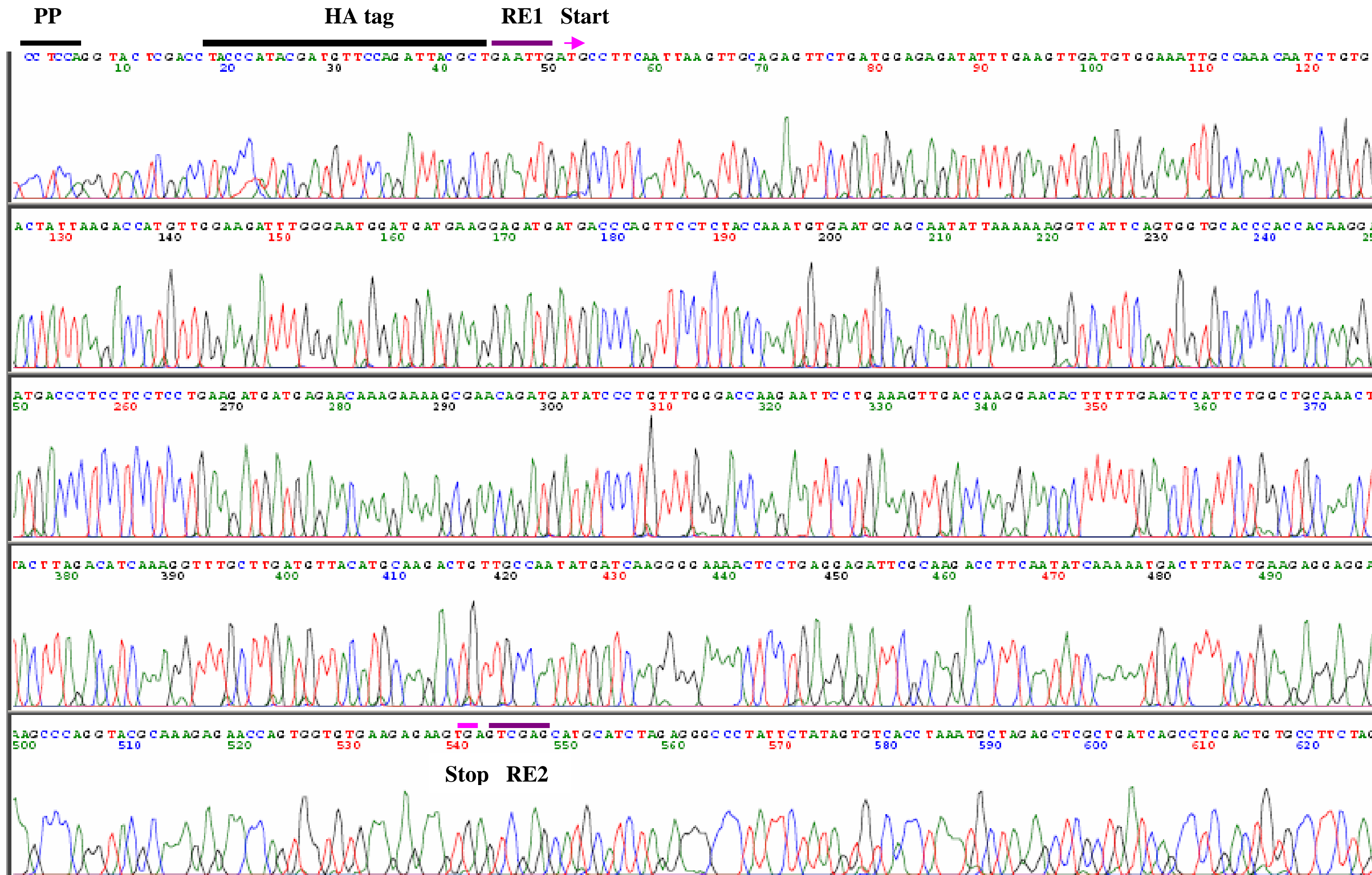
Constituent	Final concentration / 20L
Adenine sulfate	0.4g
Uracil	0.4g
Tryptophan	0.4g
Histidine	0.4g
Arginine	0.4g
Methionine	0.4g
Tyrosine	0.6g
Leucine	0.6g
Isoleucine	0.6g
Lysine	0.6g
Phenylalanine	1.0g
Glutamic acid	2.0g
Aspartic acid	2.0g
Valine	3.0g
Threonine	4.0g

*To prepare plates specific amino acid, that amino acid should be excluded from the mix. For instance, to prepare plates lacking tryptophan, no tryptophan would be added to the premix, and the amounts of all other amino acids remain the same.

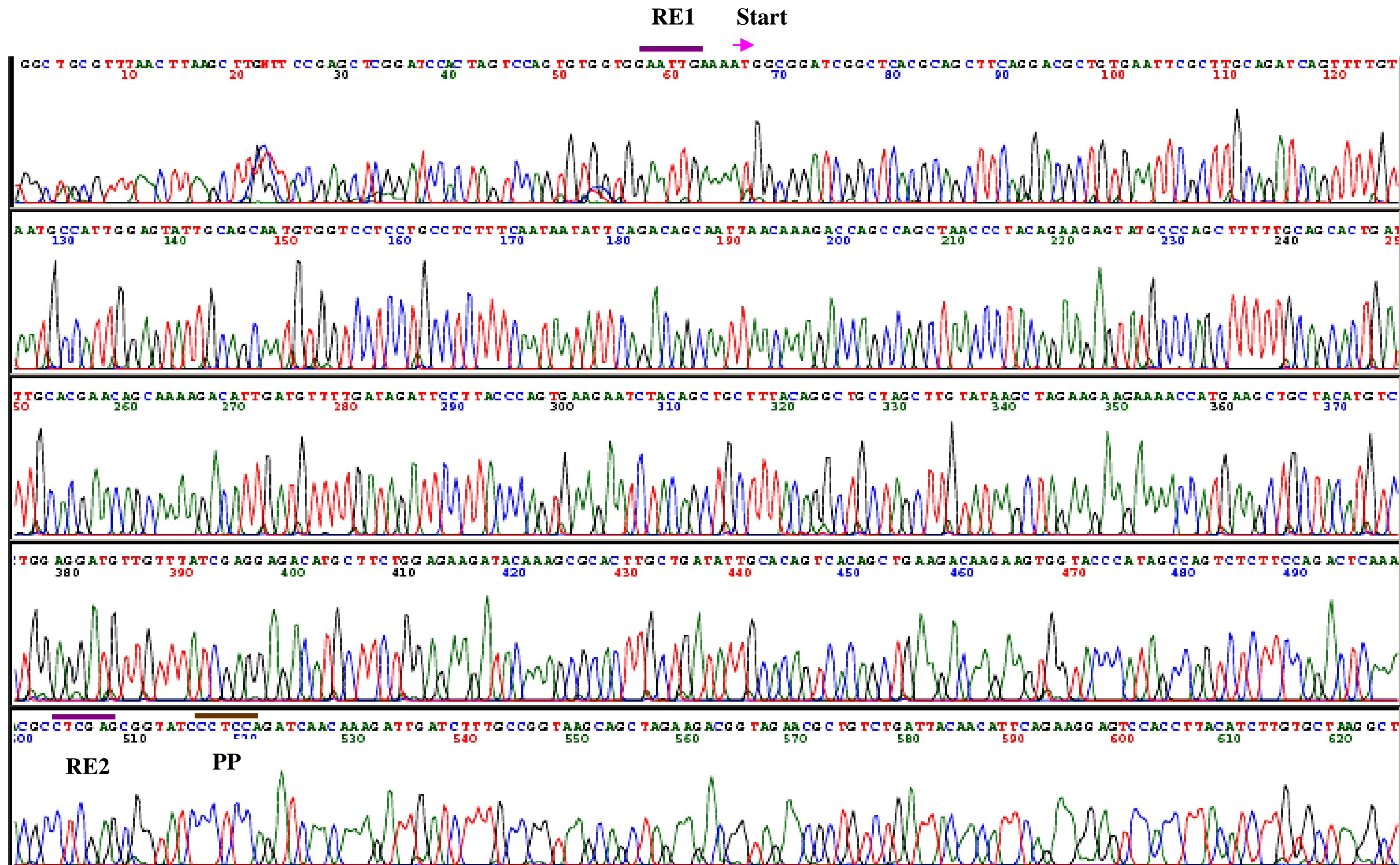
ii. Preparation of media

Consitution of premix	**Amount used /L**
Glucose	20g
Yeast Nitrogen base without amino acids	7g
Amino acid premix	0.7g

-Add 500ml of distilled water and mix before autoclaving the media.
-In another 1L bottle, weigh out 15g of Bactoagar, and add 500ml of distilled water before autoclaving it.
-Cool the premix and agar in 55°C water bath before combing the two and pouring onto plates.
-For media containing 5-FOA, add 0.85g /L. Importantly, do not autoclave the media containing 5-FOA. Instead heat at 55°C to dissolve the 5-FOA before filter sterilizing the FOA-containing remix solution into the bottle of agar. Mix well before pouring onto plates.



Appendix 3: Sequencing results of pcDNA3-N_{ub}I2-hSkp1 plasmid using the N_{ub}100 primer. The linker region between N_{ub} and hSkp1 is shown followed by the ORF of hSkp1. PP: the proline pair inserted as part of the linker region; HA tag: haemagglutinin tag used in Western blot; RE1: this site 'GAATTG' came about due to the use of *Eco*RI and *Mun*I in cloning; Start refers to the ATG start site; RE2: this site 'GTCGAG' came about due to the use of *Sal*I and *Xho*I in cloning.



Appendix 4: Sequencing results of pcDNA3-hSrb7-C_{ub}-RGpt2 plasmid using the T7 promoter primer.; RE1: this site 'GAATTG' came about due to the use of *Eco*RI and *Mun*I in the cloning; Start refers to the ATG start site; RE2: the *Xho*I site used for cloning. PP: the proline pair inserted as part of the linker region.