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Investigating the mechanisms of action of the SNF2-homolog protein Fun30

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INVESTIGATING THE MECHANISMS OF ACTION OF THE SNF2-HOMOLOG PROTEIN FUN30

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A thesis submitted for the degree of Doctor of Philosophy

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University of Dundee

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LIST OF ABBREVIATIONS

6-AU	6-Aza Uracil
ARS	Autonomously Replicating Sequence
ATP	Adenosine-5'-triphosphate
ChIP	Chromatin Immunoprecipitation
ChIP-chip	Chromatin Immunoprecipitation on a chip (microarray)
CUE	Coupling of Ubiquitin conjugation to ER degradation
d.H_2O	Deionized water/MilliQ water
DDR	DNA Damage Response
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
DSB	Double Strand Break
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene glycol tetraacetic acid
EMSA	Electrophoretic mobility shift assay
FACS	Fluorescence-activated cell sorting
GST	glutathione-S-transferase
GUB	Gal4 Upstream Binding factor
HA	Haemagglutinin
HAT	Histone Acetylases
HDAC	Histone Deacetylases
HR	Homologous recombination
HU	Hydroxy Urea
IgG	Immunoglobulin G
IR	Ionizing radiation
LA	Location Analysis
LON	Long Oligo Nucleosome
MMS	Methyl methanesulfonate
MMTV	mouse mammary tumor virus
ORC	Origin Recognition Complex
PCR	Polymerase Chain Reaction
PI	Propedium Iodide
Pi	Inorganic phosphate
RNA	Ribonucleic acid
SF2	Superfamily 2
SON	Short Oligo Nucleosome
TAP	Tandem Affinity Purification
TCA	trichloroacetic acid
TLC	Thin Layer Chromatography
YEATS	Domain interacts with chromatin modification, name derived from 5 first proteins discovered containing it (Yaf9, ENL, AF9, Taf14, Sas5)

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Abstract

In eukaryotes, the manipulation of chromatin structure represents a means of regulating access to genetic information. One way in which this can be achieved is via the action of ATP-dependent chromatin remodelling complexes related to the *S.cerevisiae* Snf_2 proteins. The Fun30 protein (Function unknown now 30) shares sequence homology with the Snf2 family of chromatin remodelling proteins. Here the activities and roles of Fun30 have been investigated further. In this study, Fun30 was found to exist predominantly as a homodimer with an apparent molecular weight of 250 KDa. Fun30 binds to DNA, mononucleosomes and nucleosomal arrays. Moreover, Fun30 was shown to cause ATP-dependent alterations to nucleosome structure. This can involve increasing the accessibility of DNA, octamer displacement by sliding *in cis* or transfer to separate DNA molecules. However, Fun30 was shown to be proficient in catalysing the exchange of histone dimers.

Deletion of *fun30* was observed to increases resistance to UV, IR and 6-azauracil raising the possibility that this protein has functions in DNA repair. Deletion of *fun30* was also found to result in temperature sensitivity. At the non permissive temperature cells were found to accumulate in the S-phase of the cell cycle. Under these conditions Rad53 was found to be phosphorylated which is consistent with the activation of the S-phase checkpoint. In order to investigate the role of Fun30 in DNA replication samples were prepared to map the genome-wide locations of Fun30 at different stages in the cell cycle. Preliminary analysis of this data suggests that there is a transient enrichment of Fun30 protein within the vicinity of replication origins during S-phase.

ChI. Introduction

1. Chromatin structure

In eukaryotic cells nuclear DNA is packaged into protein/DNA structures called chromatin. This packing in human cells involves folding of approximately 2m of linear DNA (yeast genome size estimated to be 5,770 genes or 12,136,020 bp which is equivalent to 4.08 mm in length) into the confined structure of the nucleus. The compaction of the genome is achieved in part by the interaction of DNA with histone proteins, which both compress DNA and provides an additional means of regulating access to DNA. The basic unit of chromatin is the nucleosome.

1.1. Nucleosome

During the 1970s findings by a number of researchers defined the nucleosome as the fundamental subunit of chromatin (Kornberg, 1974; Kornberg, 1977). Nucleosome core particles consist of 147 base pairs of DNA wound in 1.7 turns of left-handed superhelix about a central histone protein core (Richmond et al., 1984; Luger et al., 1997). The basic conformational structure of chromatin is described as beads on a string based on the electron microscopy observation by Olins and Olins, (1974). The 'beads' are the compact Nucleosome Core Particle (NCP). NCP is composed of eight histone proteins; two of each H2A, H2B, H3 and H4) which are joined by short stretches of free 'linker' DNA to compose the repeating 'nucleosome' unit. The four core histones interact specifically to form H2A/H2B and H3/H4 heterodimeric pairs via a conserved dimerization interface known as the histone fold. The histone fold consists of a 3-4 turn α helix, a loop of 7-8 amino acids, a longer central 8 turn α helix, a loop of 6 amino acids and a final 2-3 turn α helix. Each histone also contains histone fold extensions, which are less evolutionarily conserved and non-structured lysine rich tail domains that extend past the histone core. Each histone heterodimer is

capable of interacting with other heterodimers, but only in two specific combinations. Histones dimerize through their long α2 helices in an anti-parallel orientation, and in the case of H3 and H4, two such dimers form a 4-helix bundle stabilized by extensive H3-H3' interactions. The H2A/H2B dimer binds onto the H3/H4 tetramer via interactions between H4 and H2B which include the formation of a hydrophobic cluster (Luger et al. 1997). The histone octamer is formed by a central H3/H4 tetramer sandwiched between two H2A/H2B dimers. The histone octamer with 147 bp of DNA wrapped around it is referred to as the Nucleosome Core Particle. Crystal structures of chicken nucleosomes, fruit fly and budding yeast reveal that the overall structure of the core particle is highly conserved consistent with the high degree of conservation among the histone proteins (Harp et al., 2000; White et al., 2001; Clapier et al., 2008;).

1.1.1. DNA-histone interaction

The nucleosome contains over 120 direct protein-DNA interactions that are not spread evenly about the octamer surface but rather located at discrete sites (Luger and Richmond 1998). To illustrate the interaction of the DNA around the octamer, superhelix location (SHL) numbering can be used to define any location about the nucleosomal DNA helix. Near the sequence pseudodyad the entry and exit points of the DNA strands occur and are defined to be SHL 0, which then increases one unit every time the major groove faces up the octamer to +7 in one direction and to -7 along the other. There are two types of DNA binding sites within the octamer; the α1α1 site which uses the α1 helix from two adjacent histones and the L1L2 site formed by the L1 and L2 loops. Salt links between both side chain basic and carboxyl groups and Hydrogen bonding between main chain amides with the DNA backbone phosphates form the bulk of interactions with the DNA. This is important

given that the ubiquitous distribution of nucleosomes along genomes requires it to be a non sequence specific DNA binding factor. In addition, non-polar interactions are made between protein side chains and the deoxyribose groups, and an arginine side chain intercalates into the DNA minor groove 10 out of the 14 times it faces the octamer surface (Luger et al. 1997). The distribution and strength of DNA binding sites about the octamer surface distorts the DNA within the nucleosome core. The DNA is non-uniformly bent and also contains twist defects. The pitch of free B-form DNA in solution is 10.6 bp per turn, however, the overall twist of nucleosomal DNA is only 10.2 bp per turn, varying from a value of 9.4 to 10.9 bp per turn (Luger et al. 1997).

1.1.2. Nucleosome positioning

The interactions between histones and DNA involve the DNA backbone rather than bases and so are unlikely to be sequence specific. Nucleosomes can be assembled onto more or less any DNA sequence (Davey et al., 2002; Luger et al., 1997). However, the requirement for DNA to be wrapped around the histone octamer has the potential to provide a preference for DNA flexibility or curvature most compatible with the histone octamer (Satchwell et al., 1986). Indeed it has been observed that nucleosomes are often assembled on some sequences in preference to others. For example, a model designed using motifs identified from DNA sequences associated with budding yeast and chicken nucleosomes remarkably predicted half of the yeast nucleosomal positions *in vivo* (Segal et al., 2006). This showed that genomic DNA sequence provides a large contribution to the positioning of nucleosomes. This intrinsic positioning likely aids the formation of particular chromatin domains such as promoter regions (Ioshikhes et al., 2006; Segal et al., 2006). Moreover, a recent study showed that specific DNA-binding proteins are the

predominant determinants of chromatin architecture at the GAL1/10 genes (Floer et al., 2010). On the other hand, a recent study indicated that intrinsic histones are not the major determinants of nucleosome positioning *in vitro* (Zhang et al., 2009; Zhang, 2010). Interestingly, in this study nucleosomes assembled *in vitro* were found to have limited preference for specific translational positions and do not show the pattern observed *in vivo*. These findings argued against a genomic code for nucleosome positioning and suggest that the nucleosomal pattern in coding regions results from statistical positioning from a barrier near the promoter that involves some aspects of transcriptional initiation by RNA pol II (Zhang et al., 2009). Moreover, it is worth mentioning here that there is a difference between the term nucleosome 'occupancy' and 'positioning'. Occupancy could be defined as a measure of histone or nucleosome density and it is typically measured in genomic scale using microarrays or deep sequencing (Kaplan et al., 2009). Whereas positioning is a measure of the extent to which the population of nucleosomes resists deviation from its consensus location along the DNA and can be thought of in terms of a single reference point on the nucleosome (Albert et al., 2007).

1.1.3. Chromatin dynamics

Chromatin structure is not static but has been observed to undergo a number of different structural re-arrangements. For example, it has been shown that nucleosomes reconstituted onto the 5S DNA positioning sequence are able to reposition themselves translationally onto adjacent sequences when subjected to thermal incubation (Pennings et al. 1991). Subsequently, it was found that this observation is a general property of nucleosomes assembled onto many different DNA sequences and not just the 5S DNA (Meersseman et al. 1992), involving the redistribution of nucleosomes between a series of favored locations (Flaus and

Owen-Hughes 2003). There are two models explaining how the energetic cost of breaking all of the histone-DNA contacts simultaneously during repositioning could be avoided; either bulge diffusion or twist defect diffusion (Flaus and Owen-Hughes 2003). Despite the support for both mechanisms, it remains unclear exactly how nucleosomes reposition themselves. The ability of nucleosomes to slide along DNA may assist chromatin in the apparently contradictory tasks of condensing DNA while maintaining access for DNA binding proteins.

Work from the Widom laboratory has shown that nucleosomal DNA is in equilibrium between wrapped and partially unwrapped states (Anderson and Widom, 2002). Measurements of these rates using time resolved FRET revealed that DNA within the nucleosome remains fully wrapped for only 250ms before it is unwrapped for 10-50ms and then rapidly rewrapped (Li et al. 2005). Binding of transcription factors can act to bias this equilibrium in favor of the unwrapped state and in this way the binding of unrelated transcription factors has the potential to result in cooperative binding (Anderson et al., 2002; Li et al., 2005; Li and Widom, 2004). Another spontaneous transition in chromatin structure is the ability of arrays of nucleosomes to spontaneously fold into condensed fibres. This is discussed further below.

1.2. Higher ordered chromatin organization

The packaging achieved by the nucleosome core particle alone is not sufficient to enable the entire DNA that makes up a genome to be packaged within a nucleus. In fact it is likely that several additional layers of compaction are required to enable DNA to fit within the confines on the nucleus. After the nucleosome, the next level of organization characterized comprises a 30nm fibre made up of nucleosomes. The

structural details of the 30-nm chromatin fibre remain controversial. Nonetheless, the nucleosome chain has been long assumed to form a 30-nm chromatin fibre before the higher order organization of interphase nuclei or mitotic chromosomes. It has been demonstrated that the binding of histone H1, a chromatin associated protein promotes the folding of nucleosomal arrays into 30 nm fibres (Thoma and Koller, 1977; Thoma et al., 1979). Moreover, it has been reported that H1linker histone might have a combinatorial role in functioning both as primary chromatin architectural proteins and simultaneously as recruitment hubs for proteins involved in accessing and modifying the chromatin fiber (McBryant et al., 2010).

During the past years the 30nm fibre was explained by two significantly different models (Robinson and Rhodes, 2006). One model is derived from the crystal structure of a four-nucleosome core array lacking the linker histone (Schach et al., 2005). The other which is more compact in structure was derived from electron microscopy analysis of long nucleosome arrays containing the linker histone (Robinson and Rhodes, 2006). The first model is of the two-start helix type, the second a one-start helix with interdigitated nucleosomes. It is thought that these models provide new evidence about the topology and compactness of the '30 nm' chromatin fibre structure (Robinson and Rhodes, 2006)**.** Very recently, Grigoryev et al. showed that these two modes may be present simultaneously under certain conditions (Grigoryev et al., 2009). Beyond the 30 nm fibre chromatin is condensed further to form100-300 nm fibres. These fibres must be further condensed to eventually form mitotic chromosomes. The manner in which this is done is not yet fully understood. In an attempt to define the molecular arrangement of nucleosomes in these structures, researchers have had to depend on electron microscopy and structure predictions. Electron microscopy and sedimentation studies have shown

that chromatin condenses as ionic strength increases. It has been observed that condensation of chromatin fibres requires modest 1- 2 mM divalent ion concentration even though the gross cellular concentrations of Ca^{2+} and Mg^{2+} ions are 4-6 mM and 2-4 mM respectively suggesting that chromatin exists as a condensed form *in vivo*. Moreover, Tumbar and colleagues used bromodeoxy-uridine 5'phosphate incorporation to show that that 100 nm fibres are transcriptionally active (Tumbar et al., 1999). However, these observations are somewhat controversial and in yeast there is evidence for the compaction of chromatin at the level of the 30 nm fibre and below indicating that that active yeast chromatin is not as compact as a canonical 30 nm fibre (Dekker, 2008). Thus, it remains possible that there multiple different ways in which nucleosomes can be condensed to form chromatin fibres.

1.3. Strategies for regulating chromatin structure

All eukaryotes possess an assortment of mechanisms that act to manipulate chromatin structure. These include enzymes that act to post-translationally modify histone proteins, ATP-dependent chromatin remodelling enzymes that non-covalently alter the structure of chromatin (Viginali et al., 2000; Johnson et al., 2005; Jenuwein and Allis, 2001), histone chaperones, histone variants, DNA methylation (in some species), and the recruitment of nucleosome binding proteins such as linker histones. These are discussed in more detail below.

1.3.1. Histone modifications

Covalent chromatin modifications include; Acetylation, Methylation Ubiquitination, Sumoylation, Phosphorylation and ADP-ribosylation. Each of these modifications is catalyzed by a specific family of enzymes. For example, histone acetylation is directed by histone acetyltransferases and histone methylation is carried out by

histone methyltransferases. The identification of enzymes responsible for chromatin modification has illustrated the extent to which chromatin modifications are integrated into normal gene regulatory pathways. For example, when the tetrahymena Gcn5 protein was identified as a histone actyltransferase, it provided a new link between histone acetylation and gene regulation (Brownell et al., 1996; Jeanmougin et al., 1997). Gcn5 was already known to be a transcriptional coactivator interacting with both sequence specific transcription factors and components of the basal transcription apparatus (Jeanmougin et al., 1997). Subsequently, a diverse range of chromatin modifying enzymes has been linked to gene regulatory processes.

In general chromatin-modifying enzymes are large proteins, normally comprising more than 1000 amino acids, that have a complex multi-domain structure in which both catalytic and chromatin-binding domains play a key role (Peterson and Laniel, 2004; de la Cruz et al., 2005; Cheng et al., 2005). Frequently these proteins are found as components of multi protein complexes. For example, many of the HAT's that have been identified to date exist as components of large multisubunit protein complexes such as SAGA and NuA4.

Histone modification is a reversible process. In general enzymes removing modifications have been found to act in pathways that oppose those involved in placing the modification. For example histone deacetyltransferases (HDACs) reverse the action of acetylase transferases (HATs) and deubiquitilases oppose ubiquitin ligases and so on.

A synthetic view of the biological role of histone modifications has been given in the histone code hypothesis (Strahl and Allis, 2000; Turner, 2000; Gardner et al., 2011), according to which the regulatory state of a gene is a function of these modifications, and their combinations. Chemical modifications of histones may

affect the structure of chromatin, closing certain DNA regions or exposing others for interaction with the transcription machinery; these changes have a profound influence on gene expression (Peterson and Laniel, 2004). Alternatively, modifications or combinations of modifications may act as epitopes for the recruitment of chromatin binding proteins.

1.3.1.1. Acetylation

Histone acetylation is probably the most well studied histone modification mechanism. The attachment of an acetyl group to lysine neutralizes the basic charge of the residue. Hence, lysine acetylation could directly disrupt intra- and internucleosomal interactions. The regulation of the level of acetylation is maintained by the opposing actions of HATs and Histone Deacetylases (HDACs).

Over 40 years ago it was first proposed that there could be a connection between histone acetylation and the regulation of transcription (Allfrey, 1977). Subsequently a number of different studies established correlations between acetylation levels and gene activity (Hebbes et al., 1988; Robinson et al., 2008; Shogren-Knaak et al., 2006). For instance, it has been reported that the incorporation of the acetylation of histone H4 on lysine 16 (H4-K16Ac) inhibits the formation of compact 30-nanometer–like fibres and impedes the ability of chromatin to form cross-fibre interactions (Shogren-Knaak et al., 2006). H4-K16Ac also inhibits the ability of the adenosine triphosphate–utilizing chromatin assembly and remodelling enzyme ACF to mobilize a mononucleosome, indicating that this single histone modification modulates both higher order chromatin structure and functional interactions between a nonhistone protein and the chromatin fibre.

There are different ways via which histone acetylation has been proposed to exert its effect on transcription. For example it has been demonstrated that acetylation can be

recognized by the bromodomain motif (Dhalluin et al., 1999). These motifs are found within transcription factors, HATs and ATP-dependent remodelling enzymes (Eisen et al., 1995; Jeanmougin et al., 1997). Certain bromodomains have been found to recognize specific acetylation at specific sites, whereas other bromodomains demonstrate a more general recognition of acetylated residues (Hassan et al., 2007; Kanno et al., 2004). Moreover, it has been recently reported that the tandem plant homeodomain (PHD) finger of human DPF3b, which is involved in gene activation has also been reported to bind to acetylated histones (Zeng et al., 2010).

Genome-wide studies showed that actively transcribed genes are enriched for H3 and H4 acetylation (Brinkman et al., 2006; Liu et al., 2005; Pokholok et al., 2005). This suggests that HATs function as transcriptional activators. In yeast the SAGA and TFIID HAT complexes are involved in the regulation of ~99% of genes (Huisinga and Pugh, 2004). There are different means by which the action of HAT's may be directed to specific regions of the genome. For example, it has been shown that NuA4 and SAGA can be recruited by acidic activators such as VP16, AH, GCN4, GAL4, and HAP4 (Utley et al., 1998). Many HATs also contain bromodomains and so an alternative means of recruitment is via histone modifications. Histone acetylation does not only influence transcription, for instance H3 K56 acetylation is involved in the DNA damage response, with disruption to either K56 acetylation or deacetylation causing increased sensitivity to DNA damaging agents and growth defects in yeast (Celic et al., 2006; Chen et al., 2008; Driscoll et al., 2007; Han et al., 2007; Hyland et al., 2005; Maas et al., 2006; Masumoto et al., 2005; Recht et al., 2006). Moreover, histone H3 lysine 56 acetylation (H3K56 Ac) has recently been identified and shown to be important for genomic stability in yeast. It has been

shown that H3K56 acetylation requires the histone chaperone Asf1 and occurs mainly at the S phase in unstressed cells. Moreover, SIRT1, which is a member of sirtuin family of NAD (+)-dependent deacetylases, regulates the deacetylation of H3K56 (Yuan et al., 2009).

Additionally, it has been shown that deacetylation of H3 K56 is necessary for heterochromatin compaction at telomeres (Xu et al., 2007). Repressed telomeric regions in *Saccharomyces cerevisiae* are characterized by histone hypoacetylation, this is achieved in part by the HDAC capabilities of the Sir2 silencing protein (Braunstein et al., 1993; Imai et al., 2002; Kimura et al., 2002: Liou et al., 2005).

1.3.1.2. Histone Phosphorylation

Recently, important advances have been made towards understanding the function of histone phosphorylation in several vital processes including transcription, DNA repair, and apoptosis (Cheung et al., 2000). It is not clear yet the mechanism by which phosphorylation contributes to transcriptional activation. It is possible that the negatively charged phosphate groups neutralize the basic charge on the histone tails and as a result reduces their affinity for DNA in a similar way to that proposed for acetylation. Alternatively, histone acetylation may act to create epitopes recognized by phospho-specific interaction partners such as 14-3-3 proteins. For example, Histone H3 S10 and S28 phosphorylation are specifically recognized by the 14.3.3 proteins (beta, gamma, epsilon, sigma, zeta, tau and eta; Macdonald et al., 2005).

Phosphorylation of serine 10 in H3 has been reported to be associated with gene activation in mammalian cells and with the stimulation of transcription during heat-shock response in *Drosophila* (Thomson et al., 1999: Nowak and Corces, 2000). Moreover, mitotic chromosomes require Serines-10, -28 and theronine -11 on H3 to

be phosphorylated prior to condensation. This phosphorylation is carried out by mitosis specific kinases which are members of the Ipl1/Aurora kinase family (Goto et al., 1999; Hendzel et al., 1997; Preuss et al., 2003; Van Hooser et al., 1998). It has been established that modification of chromatin proteins such as histone H3 by phosphorylation and/or acetylation concomitant with gene activation (Clayton and Mahadevan, 2003). It is of interest to note that H3 S10 phosphorylation augments Gcn5 specificity for H3 K14 acetylation (Cheung et al., 2000), whereas T11 phosphorylation increases Gcn5 acetyltransferase activity towards H3 K9 (Shimada et al., 2008). Histone H3 peptides phosphorylated at either site were preferentially bound by Gcn5 relative to unmodified peptides (Cheung et al., 2000; Lo et al., 2000).

Phosphorylation of histone H3 has also been shown to occur after activation of DNA-damage signaling pathways suggesting that phosphorylation mediates modifications of chromatin structure, which in turn assists repair (Downs et al., 2004). During DNA damage, CHK1 dissociates from promoter chromatin, causing a reduction in T11 phosphorylation and K9 acetylation and repression of transcription (Shimada et al., 2008). Another important phophorylation event is the H2A phosphorylation, which will be discussed in paragraph 1.4.3.1. Chromatin alterations and DNA repair. Taken together, all these findings suggest that histone phosphorylation play diverse number of roles throughout the cell cycle.

1.3.1.3. Methylation and chromatin regulation

In 1964, methylation of histones was described for the first time (Murray, 1964). Thirty five years later, definite evidence linking methylation and transcription was found, when the histone H3 arginine-specific histone methyltransferase (HMT)

CARM1 was revealed to interact with the steroid-hormone-receptor coactivator GRIP-1 in transcriptional initiation (Chen et al., 1999). Preliminary studies of the sites at which histones are methylated indicated that histones H3 (lysines 4, 9 and 27) and H4 (lysine 20) were predominant (Strahl et al., 1999). These modified lysines could be mono-, di- or tri- methylated, adding an additional complexity to the posttranslational status of H3 and H4 tails. Methylation of H3 K9, K27 and H4 K20 has been linked with repressed chromatin structures. H3 K9 methylation is directed by the human SUV39H1, murine Suv39h1 and its homologs in other species (Rea et al., 2000; Nakayama et al., 2001). H3 K9 methylation has in turn, been shown to promote heterochromatin formation via recruitment of HP1 through its chromodomain (Bannister et al. 2001; Lachner et al. 2001), (Nielsen et al. 2002; Jacobs and Khorasanizadeh, 2002). However, not all chromodomains bind methyl-lysines (Bouazoune et al. 2002), and the interaction of HP1 with chromatin *in vitro* appears to be influenced via the hinge domain as well as the chromodomain (Meehan et al. 2003).

On the other hand, methylation at other sites is linked to transcriptional activation. Methylation of H4 R3 has been shown to promote H4 acetylation and transcriptional activation (Wang et al.,2001), yet, until now, no dedicated methyl-arginine binding modules have been identified.

1.3.1.4. Ubiquitination

Ubiquitin (originally, Ubiquitous Immunopoietic Polypeptide) was first identified in 1975 as an 8.5-kDa protein of unknown function expressed commonly in living cells (Hu et al., 2002 and Ciechanover et al., 1978). The ubiquitination system was originally characterized as an ATP-dependent proteolytic system present in cellular extracts (Hu et al., 2002). Many important cellular processes such as transcription

and cell cycle regulation are regulated through posttranslational modification by ubiquitin (Hochstrasser 1996; Hershko et al. 2000; Pickart, 2001). The Ubiquitin prototypically recognizes specific protein substrates and places polyubiquitin chains on them for subsequent destruction by the proteasome (Willis et al., 2010). This system is in place to degrade not only misfolded and damaged proteins, but is essential also in regulating a host of cell signaling pathways involved in proliferation, adaptation to stress, regulation of cell size, and cell death (Willis et al., 2010). Ubiquitination is carried out by a set of three enzymes, E1, E2 and E3 (Willis et al., 2010). Ubiquitin is first activated by ubiquitin-activating enzyme E1, after that the ubiquitin molecule is then passed on to the second enzyme of the complex, E2 (ubiquitin-conjugating enzyme), before reaching the final enzyme, E3, the ubiquitin protein ligase, which recognizes, binds the target substrate and labels it with the ubiquitin (Willis et al., 2010).

It is now clear that proteins can be initially conjugated to a single ubiquitin moiety. This can serve as an active signal in itself or as a substrate for polyubiquitin conjugation (Pickart, 2000). Monoubiquitin conjugation plays a significant role in modulating numerous cellular pathways, including transcription regulation, histone modification, and protein trafficking in the endocytic pathway (Hicke, 2001). For instance, the carboxyl terminus of histone H2B is ubiquitinated in yeast in a Rad6-dependent fashion and the loss of this ubiquitination site has been found to result in defects in mitosis and meiosis (Robzyk et al., 2000). Several protein domains interacting with monoubiquitin and/or promoting monoubiquitination had been recently identified. These domains include ubiquitin-associated motifs (UBA), ubiquitin interacting motifs (UIM), and more recently, the CUE motif, named after

the yeast Cue1 protein (Coupling of Ubiquitin conjugation to *ER* degradation). CUE motifs are approximately 40 amino acids in length and are found throughout many eukaryotic proteins (Ponting, 2000).

Ubiquitination of histones is correlated with transcriptional activation, and Ubiquitination of H2B K119 has been found to be required for methylation of H3 K4 and K79 methylation (Briggs et al. 2002; Sun and Allis 2002). It is not currently known whether histone ubiquitination induces large scale alterations in chromatin structure or whether it is able to activate chromatin independently of its effect on H3 K4 and K79 methylation.

1.3.1.5. Other modifications

A significant number of other histone modifications within the unstructured tails and in the globular core of histones have not been described here. These include ribosylation, sumoylation, biotinylation, and adenylation and proline isomerisation. The latest discoveries regarding these histone modifications and the enzymes that catalyze them have indicated a significant interaction between several covalent marks and the proteins that identify them (Strahl and Allis, 2000; Bártová et al., 2008). However, many of the basic principles underlying the ways in which they act are conceptually similar to those described above for acetylation, methylation, ubiquitination and phosphorylation.

1.3.2. Histone variants

In addition to the major histone species within cells a series of minor histone proteins termed variants has been discovered. Histone variants are encoded by distinct, nonallelic genes (Redon et al., 2002). These variants not only demonstrate sequence differences but are also expressed at different times. The incorporation of the histone

variants into nucleosomes acts to specify chromatin for particular biological roles (Malik and Henikoff, 2003; Talbert and Henikoff, 2010). Some studies have indicated that variants of histone H2A and histone H3 play significant roles in gene expression, the repair of DNA breaks and the assembly of chromosome centromeres (Smith, 2002; Redon et al., 2002;).

H2AZ is one of the first discovered variants and is present in nearly all eukaryotes. In mammals, H2AZ is essential (Faast et al. 2001), whilst in yeast it is not; but its deletion increases the need for chromatin remodelling enzymes to promote transcription (Santisteban et al. 2000). H2AZ is required for the expression of genes that cluster near the sub-telomeric region and it has been proposed to act as a boundary element to stop the spread of heterochromatin (Meneghini et al. 2003).
H2AX is similar to canonical H2A and is involved in the repair of double stranded breaks, and upon DNA damage is rapidly phosphorylated on the serine within the C-terminal motif (Downs et al. 2004; Morrison et al. 2004). In yeast there is no histone H2AX variant, but the major form of H2A is phosphorylated in a similar way and fulfilling its role. (Shroff et al., 2004; Lydall and Whitehall, 2005).

H3.3 is remarkably similar to canonical H3, with only 4 amino acid differences between the *Drosophila* histones. Whereas expression of major H3 is limited to S phase and is replication coupled (RC), H3.3 is expressed in a replication independent (RI) manner (Ahmad and Henikoff 2002). H3.3 is the main form of H3 at actively transcribed regions of the genome and is consequently enriched in modifications associated with active chromatin (McKittrick et al. 2004). CENP-A is a centromere specific form of H3 and mice lacking it die early in development of severe mitotic

defects (Howman et al. 2000). The association of the human protein CENP-A with H4 into tetramers reveals differences in intermolecular packing compared with canonical H3/H4 as is both more compact and rigid (Black et al. 2004).

1.3.3. Nucleosome binding proteins – linker histones and HMG proteins

There are a range of abundant basic proteins that can bind to nucleosomes and have the potential to alter the function of chromatin. For example, linker histones such as histone H1 are known to bind nucleosomes to form chromatosomes (Thomas, 1999; Bharath et al. 2003). The association of linker histones with nucleosomes has been shown to influence chromatin fibre formation (Hizume et al. 2005; Carruthers et al. 1998; Bednar et al. 1998; Thoma et al. 1979). Moreover, there is evidence that H1 subtypes can differentially affect gene expression *in vivo* and that some may stimulate it (Alami et al. 2003). However, it should be noted that in budding yeast mutation of the linker histone is associated with minor phenotypes.

Other proteins that can associate with nucleosomes include High mobility group (HMG) proteins. HMGs are nuclear proteins that affect the packaging of the chromatin fibre and regulate gene expression by altering the ability of regulatory factors to access their binding sites (Bustin et al., 2005; Catez et al., 2004; Catez et al., 2002). HMG are divided into three classes of proteins: HMGA (formerly HMGI/Y and HMGI−C), HMGN (formerly HMG14 and HMG17), and HMGB (formerly HMG1 and HMG2) (Bustin, 1999). HMGN members are the only proteins that bind specifically to nucleosomal particles (Bustin, 2001). HMGN proteins are able to bind tightly to nucleosome core particles and interact both with H2B and the H3 tail (Trieschmann et al., 1998). HMG proteins decrease the compactness of the

chromatin whereas H1 histones alleviate the higher order chromatin structure and limit nucleosomal access. Both HMG proteins and linker histones are very rich in proline residues which may contribute to the unstructured or partly structured nature of these proteins.

1.3.4. Histone Chaperones

Histones are not always present within cells in the context of nucleosomes. For example, the bulk of newly synthesized histones must be transported to the nucleus and targeted to sites of DNA replication (Woodland and Adamson, 1977; Jasencakova et al., 2010). In addition histones can be transiently removed from DNA during histone eviction and replacement. Since histones are highly basic proteins, their presence in the cell could lead to detrimental effects. This could be the reason why histones not bound by DNA are normally found in association with other proteins termed histone chaperones (Loyola and Almouzni, 2004). Histone chaperones could be classified based on their structure, biological function and types of histone they transfer. One of the recent classifications group histone chaperones into five main structural categories (a) β-sandwich structures such as the Asf1 and Yaf9 YEATS domain containing proteins **(b)** α/β-earmuff histone chaperones such as NAP1 and Vps75 **(c)** β-propeller chaperones such as the structures of Np and the RbAp46–H4 complex **(d)** β-barrel and half-barrel chaperones such as Pob3 (a FACT subunit) and Rtt106 **(e)** Irregular such as Chz1 (Das et al., 2010).

Activities of various enzymes including ATP-dependent chromatin remodelers (ATPases) and histone chaperones were considered crucial to abrogate the nucleosomal barrier (Tyler, 2002). For example, RSC (Remodels Structure of Chromatin) alone stimulates elongation through the nucleosome, but depends on an

acceptor DNA molecule to receive the octamer evicted from the transcribed template (Lorch et al., 2006; Lorch et al., 2010). It has been found that Nap1 stimulates RSC-dependent transcription in the absence of an acceptor DNA molecule. These data support a model where RSC disassembles a nucleosome in the presence of the histone chaperone Nap1 and ATP. Disassembly occurs in a gradual manner, with the removal of H2A/H2B dimers, followed by the rest of the histones and the release of naked DNA (Lorch et al., 2006). Alternatively, the FACT (facilitates chromatin transcription) complex can promote RNA pol II transcription through nucleosomal templates in an ATP-independent manner (Orphanides et al., 1998). FACT has also been demonstrated to remove dimers from nucleosomes during transcription (Belotserkovskya et al., 2003). Moreover, it has been demonstrated that position-dependent gene silencing in yeast involves many factors, including the four HIR genes and histone chaperons Asf1p and chromatin assembly factor I (CAF-I, encoded by the CAC1–3 genes) (Sharp et al., 2001). Notably, CAF-1 is not associated with the replacement histone variant H3.3, which is incorporated into chromatin independently of DNA replication. CAF-1 is targeted to sites of DNA synthesis that are associated with either replication or repair via a direct interaction with PCNA (Moggs et al., 2000).

In summary, histone chaperones impede unplanned interactions of histones with other factors and facilitate in controlling histone supply and their integration into chromatin (Loyola and Almouzni, 2004).

1.3.5. ATP dependent chromatin remodelling enzymes

All eukaryotes encode a number of enzymes that use energy derived from ATP hydrolysis to reconfigure chromatin structure. These enzymes share sequence homology with the RecA domains of Superfamily II helicase related proteins (Eissen

et al., 1995). The yeast Snf2 protein was the first of these enzymes to be identified and subsequently it has become apparent that ATP-dependent proteins can be further classified into 24 subfamilies based on their biochemical properties and the overall sequence similarity of their ATPase subunits (Flaus et al., 2006). As most of the subfamilies are conserved across a broad evolutionary scale they are each likely to have distinct functions. However, more closely related subfamilies can be organised into general groupings (see Fig. 1.1) including Snf2, Swr1, SSO1653, Rad54, Rad5/16 which are considered likely to have shared characteristics (Flaus et al., 2006). For example, the Snf2 grouping includes the Snf2, Iswi and Chd1 subfamilies share the ability to reposition nucleosomes.

It is thought that Snf2 proteins share ATP-dependent DNA translocation activity but this is generally not associated with the separation of DNA strands. There are several mechanisms by which these complexes can alter chromatin structure. For example, the SWI/SNF complex disrupts DNA-histone contacts in a way that can result in the release of histones, ISWI family remodelers act predominantly by repositioning nucleosomes along DNA and the Swr1 complex directs the incorporation of a histone variant (Cairns, 2007).While the Snf2 grouping is closely associated with chromatin remodelling especially nucleosome sliding, this is not true of all the groupings. For example, the SSO1653 grouping (which is not found in eukaryotes) includes the Mot1 subfamily that acts to displace TBP from DNA in an ATP dependent reaction (Flaus et al., 2006). Even within the enzymes more closely linked to chromatin, biological functions are still being investigated. In the following sections current understanding of some of these subfamilies will be described in more detail.

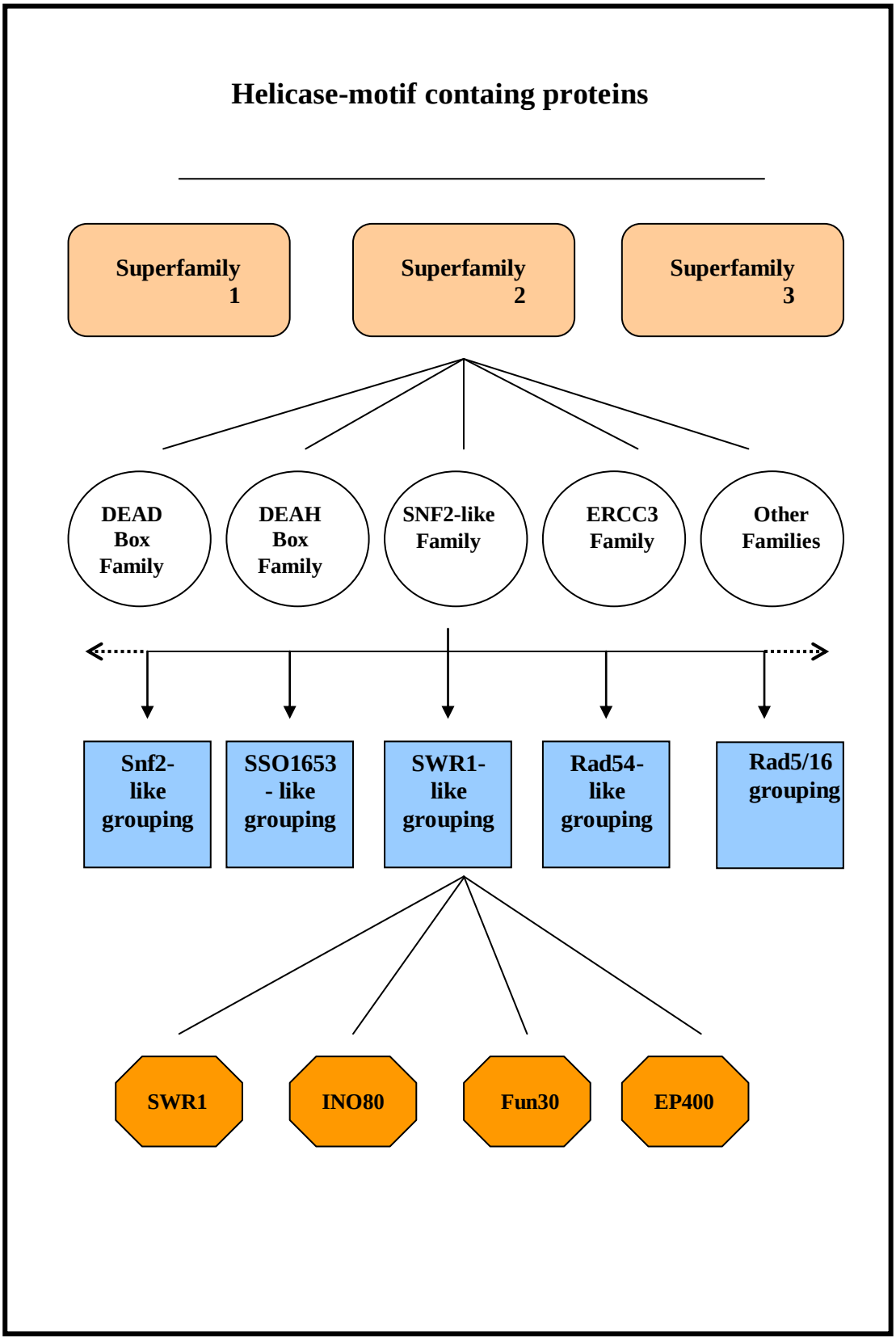

Figure 1.1: Schematic diagram of the SWR-1 subfamily

1.3.5.1. The snf2 subfamily of chromatin remodelling enzymes

The first ATP-dependent chromatin remodelling protein to be identified was the yeast (*S. cerevisiae*) Snf2 (otherwise known as Swi2) protein (Winston and Carlson, 1992). This was subsequently found to be a component of a multi-protein complex termed the SWI/SNF (mating type switching/sucrose non fermenter) complex (Peterson et al., 1994) that is capable of distorting chromatin structure in an ATP-dependent reaction (Cote et al., 1994; Tang et al., 2010). Subsequently, it has become clear that the SWI/SNF complex is conserved across a range of eukaryotes and that the genomes of most eukaryotes possess many proteins related to Snf2. The *Saccharomyces cerevisiae* Snf2p catalytic subunit is none-essential for viability (Laurent et al., 1992) and the complex is of low abundance, with only 50-150 copies per cell (Cote et al., 1994). Therefore, the action of these complexes must be targeted to specific regions of the genome. This targeting occurs both via transcription factors and chromatin modifications (Gutierrez et al., 2007; Hassan et al., 2002).

It has been revealed by the structure of the nucleosome-remodelling domain of zebrafish Rad54 (a protein involved in Rad51-mediated homologous recombination), that the core of the SWI2/SNF2 enzymes consists of two α/β-lobes similar to SF2 helicases (Thoma et al., 2005). The Rad54 helicase lobes contain insertions that form two helical domains, one within each lobe (Thoma et al., 2005). These insertions contain SWI2/SNF2-specific sequence motifs likely to be central to SWI2/SNF2 function (Thoma et al., 2005). A broad cleft formed by the two lobes and flanked by the helical insertions contains residues conserved in SWI2/SNF2 proteins and motifs implicated in DNA-binding by SF2 helicases. The Rad54 structure suggests that

SWI2/SNF2 proteins use a mechanism analogous to helicases to translocate on dsDNA (Thoma et al., 2005).

The Snf2 protein includes a bromodomain which is required for the recruitment of SWI/SNF to acetylated chromatin (Hassan et al. 2001; Hassan et al. 2002). Consistent with this genes encoding components of the SWI/NF complex have been found to genetically interact with components of the SAGA HAT complex (Roberts and Winston 1997). Many studies have focused on the active recruitment of remodelling enzymes by DNA site-specific transcriptional activators or repressors (Peterson and Workman, 2000). For example, yeast SWI/SNF interacts with the acidic activation domains of several activators, and these contacts can target remodelling activity *in vitro* and *in vivo* (Hassan et al., 2001; Hassan et al., 2002; Hassan et al., 2006; Peterson and Workman, 2000).

Snf2 subfamily remodelling enzymes have been shown to slide nucleosomes along DNA *in cis* (Whitehouse et al. 1999; Flaus and Owen-Hughes 2003), specifically, RSC (Remodels the Structure of Chromatin) and SWI/SNF have been shown to catalyze similar reactions *in vitro*. Both are able to slide nucleosomes off DNA ends by up to 50bp, resulting in H2A/H2B dimer loss (Kassabov et al. 2003; Bruno et al. 2003) and both have been shown to transfer histone octamers *in trans* (Lorch et al. 1999; Whitehouse et al. 1999). Certain remodelling enzymes are able to transfer an entire histone octamer *in trans* from a donor nucleosome to an acceptor DNA. Furthermore, several remodelling enzymes of this subfamily have been shown to generate unconstrained negative superhelical torsion in DNA and chromatin (Havas et al. 2000). Interestingly, the low resolution 3D EM models for both enzymes indicate a large central pocket large enough to accommodate an entire nucleosome

(Asturias et al. 2002; Smith et al. 2003). Recently, a structure of a RSC-nucleosome complex with a nucleosome determined by cryo-EM showed the nucleosome bound in a central RSC cavity (Chaban et al., 2008). In this study, nucleosomal DNA appears disordered and largely free to bulge out into solution as required for remodeling, but the structure of the RSC-nucleosome complex indicates that RSC is unlikely to displace the octamer from the nucleosome to which it is bound (Chaban et al., 2008). This suggested that ATP-dependent DNA translocation by RSC may result in the eviction of histone octamers from adjacent nucleosomes (Chaban et al., 2008).

1.3.5.2. Chd1 chromatin remodelling subfamily

The CHD (chromodomain-helicase-DNA binding protein) subfamily members are differentiated by an ATPases that contain chromodomains. Chd subfamily can be further divided into a series of sub-groupings: Chd1, Chd2, Chd3/4 and Chd5-9, which all containing a chromodomain within the catalytic subunit (Eisen et al., 1995; Flaus et al., 2006). Budding yeast encodes a representative of only one of these enzymes, the Chd1 protein. The chromodomains of the human Chd1 protein have been demonstrated to bind di- and tri-methylated H3 K4 (Kelley et al., 1999; Flanagan et al., 2005). However, it has been shown that yeast Chd1 does not bind H3 methylated K4 (Sims et al., 2005).Very recently, it has been shown that the chromodomains regulate DNA Access to the ATPase Motor (Hauk et al., 2010). Biochemical characterization of Chd1 has shown that it preferentially interacts with nucleosomes containing linker DNA and acts to reposition nucleosomes in *cis* away from the ends of short DNA fragments (Stockdale et al., 2006). This repositioning behavior requires the H4 N-terminal tail (Ferreira et al., 2007). Chd1 is also especially proficient in generating regular spacing between nucleosomes on longer

DNA fragments (Lusser et al., 2005). In yeast, Chd1 has been shown to interact with FACT (Kelley et al. 1999; Krogan et al. 2002), and been found to play a role in transcription elongation (Simic et al. 2003), termination (Allen et al. 2002) and in responding to transcriptional stress (Zhang et al. 2005b). It has also been shown that Chd1 can associate with RNA pol II elongation factors (Krogan et al., 2002; Simic et al., 2003) and also influences DNA replication (Biswas et al., 2008). In *Drosophila,* Chd1 co-localizes with regions of high transcriptional activity (Stokes et al. 1996). More recently, Drosophila Chd1 has been shown to facilitate the deposition of the histone variant H3 (Konev et al., 2007). Consistent with roles in these fundamental processes, Chd1 proteins are implicated in a range of developmental processes. For example, in mice Chd1 has been found to be necessary for maintaining the pluripotency of embryonic stem cells (Gaspar-Maia et al., 2009).

1.3.5.3. ISWI chromatin organizers

The ISWI (Imitation of SWI/SNF) complex was identified in *Drosophila* on the basis of homology to the yeast protein Snf2p (Elfring et al. 1994). ISWI proteins have subsequently been found to act to as the catalytic subunits of an assortment of remodelling enzymes. These complexes function in a diverse range of processes including nucleosome assembly and spacing (ACF, CHRAC, and RSF), replication (WICH), transcriptional repression (NoRC), transcriptional activation (NURF and CERF) and for preserving the correct orientation of transcription along coding regions (Collins et al. 2002: LeRoy et al. 1998; Poot et al. 2004; Badenhorst et al. 2002; Strohner et al. 2004; Whitehouse et al., 2007). *S.cerevisiae* contains two ISWI homologs: Isw1p and Isw2p. Isw1p forms two complexes, Isw1a and Isw1b that are involved in the regulation of a number of loci including the MET16 gene, where they play opposing roles in its expression (Morillon et al. 2003). Isw2 is involved in the

repression of mating type loci (Goldmark et al. 2000) and the regulation of pol II transcriptional termination (Alen et al. 2002). Recently, it has been shown that ISWI also interacts with the Sin3A/Rpd3 HDAC repressor complex (Burgio et al., 2008). ISWI has the ability to alter chromatin structure in an ATP-dependent reaction. ISWI containing complexes redistribute nucleosomes to positions closely related to those observed in thermal nucleosome redistribution reactions or preferentially move nucleosomes to positions closer to the center of short DNA fragments (Stockdale et al., 2006). It has been reported that ISWI repositioning requires the H4 K16-R19 residues (Clapier et al., 2002; Hamiche et al., 2001) and linker DNA with an ideal length of ~80 bp (Dang et al., 2006). Both of which have been shown to be important to stabilize ISWI association with the nucleosomal SHL 2 location during repositioning (Dang et al., 2006; Kagalwala et al., 2004). Acetylation at H4 K12 and K16 residues reduces association of ISWI with nucleosomes (Clapier et al., 2002; Corona et al., 2002).

1.3.5.4. Histone exchangers; SWR1 subfamily

In 2003 a new activity for a Snf2 family protein was indentified, the ability to direct incorporation of a histone variant. This activity was characterised as an activity of the SWR1 (SWI/SNF-related protein) complex. Swr1 is a Swi2/Snf2-related ATPase with a split conserved ATPase domain with Swr1 as the main catalytic subunit in a 14 subunit complex. Swr1 complex replaces the histone H2A dimers with dimers containing the histone variant H2A.Z (Krogan et al., 2003; Kobor et al., 2004; Mizuguchi et al., 2004; Wu et al., 2005). The Swr1 protein possesses an increased spacing between conserved motifs III and IV, a feature that is shared by the closely related Ino80, Etl and EP-400 subfamilies (see Table.1.1). Each of these subfamilies described in more detail below.

Table1.1: Summary of SWR1 subfamily members, their homologs, subunits and functions

Subfamily	Subunits of yeast complex	Homologs	Functions
SWR1	Swr1, Swc2/Vp372, Swc3, Swc4/Eaf2/God1, Swc5/Aor1, Swc6/Vps71, Swc7,Yaf9, Bdf1, Act1/actin, Arp4, Arp6, Rvb1 and Rvb2	*Homo sapien*: SRCAP [Snf2p-related CBP activating protein] *Drosophila melanogaster*: Domino	- Deposits the histone variant dimer H2AZ-H2B into nucleosomes by replacing the existing H2A-H2B dimer - Regulate transcription by preferentially positioning histone variant H2A.Z within chromatin and aid in DNA repair.
INO80	Ino80, Rvb1(or TIP48), Rvb2 (or TIP49), Arp4, Arp5, Arp8, Actin, Nhp10 (non-histone protein 10), Anc1/Taf14, Ies1 (ino eighty subunit 1), Ies2, Ies3, Ies4, Ies5 and Ies6	*Homo sapien*: INO80 *Mus musculus*: Inoc (INO80 complex homolog)	- Reposition nucleosomes, and separate DNA strands in a traditional helicase assay. - Remodel chromatin in order to allow efficient double strand breaks (DSB) processing by nucleases.
Fun30	Fun30 and weakly interacts have been identified	*Homo sapien*: SMARCAD1 *Mus musculus*: Etl1 (smarcad1)	- Unknown till now, might be involved in DNA repair, cell cycle regulation and its overexpression affects chromosome stability. - A potential Cdc28p substrate
EP400	Not relevant as it not present on yeast	*Homo sapien*: EP400 *Mus musculus*: EP400	- Cell cycle control, apoptosis, development embryonic hematopoiesis - Recruited along with the Tip60 complex on promoters by the c-myc and E2F transcription factors. - Exchange specific histone variants at double-strand breaks. - Target for the E1A viral oncoprotein transforming activity

1.3.5.4.1. Swr1

The purified yeast SWR1 (SWI/SNF-related protein) complex contains fourteen polypeptides: Swr1, Swc2/Vp372, Swc3, Swc4/Eaf2/God1, Swc5/Aor1, Swc6/Vps71, Swc7, Yaf9, Bdf1, Act1/actin, Arp4, Arp6, Rvb1 and Rvb2 (Bao and Shen, 2007; Mizuguchi et al., 2004; Wu et al., 2005). This complex was designated SWR1-Com in reference to the Swr1p subunit, a Swi2/Snf2-paralog. The SWR1 complex was found to be able to specifically exchange histone H2A in nucleosomes for its variant H2AZ *in vitro* (Mizuguchi et al., 2004). Furthermore, genome wide ChIP-Chip analysis shows that Swr1 is enriched at Htz1 (the yeast homolog of H2AZ) containing nucleosomes and deletion of Swr1 results in the redistribution of Htz1 (Zhang et al., 2004). *swr1* and *htz1* mutants share common phenotypes, such as hypersensitivities to methyl methanesulfonate (MMS), UV Irradiation and caffeine (Mizuguchi et al., 2004). Moreover, genome-wide transcription profiles of *Δswr1* cells and *Δhtz1* cells showed that there is about 40% overlap between genes regulated by Swr1 and Htz1, indicating that Swr1 and H2AZ share a common function in regulating transcription (Mizuguchi et al., 2004). Htz1 plays an important role in acting as a boundary to prevent spreading of the Sir2 silencing complex into subtelomeric chromatin (Xu et al., 2007). As a result it is perhaps expected that Swr1 mutations also cause defects in Sir2 mediated silencing.

Interestingly, in some higher eukaryotes the functions of these complexes are combined. For instance, it has been reported that the *Drosophila melanogaster* SWR1 homolog Domino is found within a complex that combines components of the homologous yeast SWR1 and NuA4 complexes (Doyon and Cote, 2004). There is evidence that histone acetylation and deposition of Htz1 are inter related. For

example, Swr1 shares a number of subunits in common with the NuA4 HAT complex (Fig. 1.2) and also contains the bromodomain protein Bdf1 (Krogan et al. 2004). It has been found that the localization of H2A.Z within the yeast genome is affected by bromodomain protein Bdf1 deletion or mutation of acetylatible histone lysine residues (Raisner et al. 2005; Zhang et al. 2005a), suggesting that histone acetylation is likely to affect Swr1 activity. Efficient deposition of H2A.Z is further promoted by a specific pattern of histone H3 and H4 tail acetylation by the bromodomain protein Bdf1 (Raisner et al., 2005). Recently, it has been demonstrated that SWR1 resides at the heterochromatin boundary to maintain and amplify antisilencing activity of histone H4 acetylation through incorporating H2AZ into chromatin (Zhou et al., 2010).

The Swr1 complex also shares subunits with the Ino80 chromatin remodelling complex (Fig. 1.2.). For example, Arp4 is a subunit of both the INO80 and SWR1 chromatin remodelling complexes (Downs et al., 2004). As Arp4 can interact with H2A P-Ser129 this may provide an explanation as to why both complexes are recruited to double strand breaks (DSBs) (Downs et al., 2004).

The specificity of the SWR1 complex for H2A.Z is dependent on The C-terminal alpha-helix of H2AZ (Wu et al., 2005). Within the Swr1 complex, Swc2 has been identified as a subunit interacting directly with H2A.Z (Wu et al., 2005). Seven SWR1 subunits (Swr1- ATPase, Swc2, Swc3, Arp6, Swc5, Yaf9, and Swc6) has been identified to play a crucial role in maintaining complex integrity and H2A.Z histone replacement activity (Wu et al., 2009). However much remains to be learnt about the mechanism underlying ATP-dependent histone exchange.

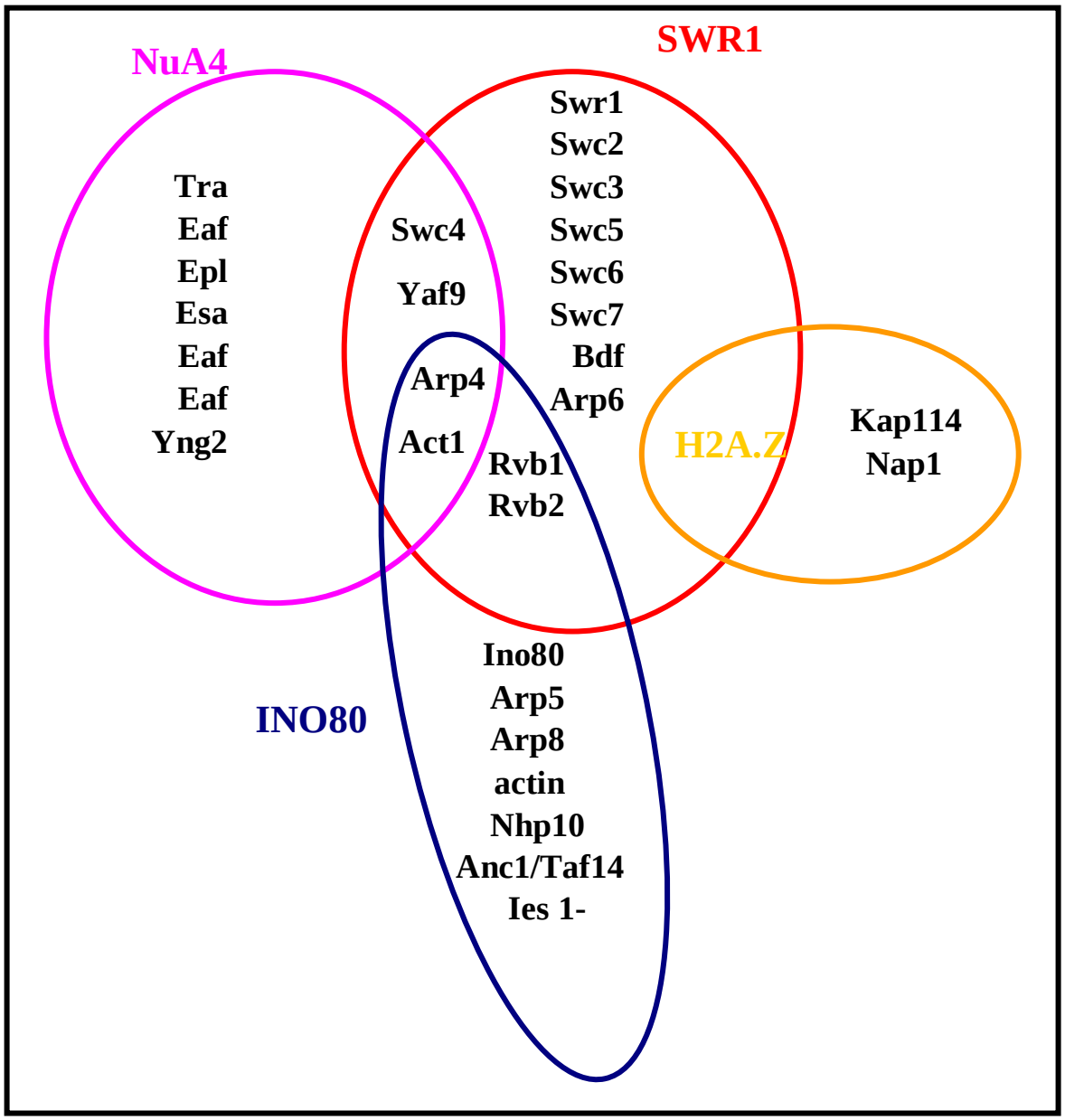

Figure 1.2: Schematic model of SWR1 and INO80 functional interactions with NuA4 and H2A.Z. (Modified from Kobor et al., 2004)

1.3.5.4.2. Ino80

Ino80 was first purified from *S. cerevisiae* by immunoprecipitation and was named as a result of its role in transcriptional regulation of inositol biosynthesis (Bachawat et al., 1995; Ebbert et al., 1999). Ino80 exists as a 15 subunit complex, INO80.com, (Bakshi et al., 2004; Shen et al., 2000). The INO80 complex is notable not only because it can reposition nucleosomes, but also because it is the only known Snf2 family-related complex able to separate DNA strands in a traditional helicase assay (Shen et al., 2000). This strand separating activity has a 3' to 5' polarity and requires the Rvb1 (or TIP48), Rvb2 (or TIP49) subunits. These proteins are both members of the AAA+ superfamily of Superfamily I helicase related proteins (Shen et al., 2000; Jonsson et al., 2001; Zophonias et al., 2004; Neuwald et al., 1999; Vale, 2000). The bacterial homologs of the Rvb1 and Rvb2 act as subunits of the RecBCD complex where they act as processive strand separating helicases. The Rvb1/2 proteins are conserved in *E.coli* where they form part of the RuvB holliday junction translocase (Shen et al. 2000).Their function within the Ino80 complex is not well defined. However, both *RVB* genes are individually essential for viability in yeast, as are their homologs in other eukaryotic organisms (Jonsson et al., 2001).

In addition to the, Rvb1 and Rvb2 subunits the Ino80 complex also contains Arp4, Arp5, Arp8, Actin, Nhp10 (non-histone protein 10), Anc1/Taf14, Ies1 (Ino80 subunit 1), Ies2, Ies3, Ies4, Ies5 and Ies6 (Mizuguchi et al., 2004; Shen et al., 2000; Shen et al., 2003). The SWR1 and INO80 complexes share three proteins (Rvb1/Tip49a, Rvb2/Tip49b, and Arp4) and contain additional homologous components (see Fig. 1.2). However, each of the two complexes has a number of unique subunits. It has been recently reported that Ino80 remodelling activity resembles the nucleosome

spacing activity of ISW2 and ISW1a, which are generally involved in transcription repression (Udugama et al., 2010). It has been shown that Ino80 required a minimum of 33 to 43 bp of extranucleosomal DNA for mobilizing nucleosomes, with 70 bp being optimal (Udugama et al., 2010). However, Ino80 does not require the H4 tail for nucleosome mobilization; instead, the H2A histone tail negatively regulates nucleosome movement by Ino80 (Udugama et al., 2010).

Mutants of INO80 show hypersensitivity to agents that cause DNA damage, in addition to defects in transcription (Shen et al., 2000). Recent studies have implicated INO80 directly in the events of double-stranded break repair. For example, components of the INO80 complex show synthetic genetic interactions with the RAD52 DNA repair pathway, the main pathway for DSB repair in yeast (Morrison et al., 2004). These findings implicate INO80-mediated chromatin remodelling directly at DSBs, where it appears to facilitate processing of the lesion (van Attikum et al., 2004). It seems possible that the INO80 complex could be involved in the eviction of nucleosomes in the vicinity of the break (Tsukuda et al., 2005) although other remodelling complexes such as Swr1, RSC and SWI/SNF may also be involved in this repair pathway (reviewed in Downs and Cote, 2005). Despite their involvement in the DNA repair process, mutants lacking SWR1 or INO80 remain checkpoint competent. In contrast, loss of NuA4-dependent histone acetylation leads to G1 checkpoint persistence, suggesting that H2A phosphorylation promotes two independent events, rapid Rad9 recruitment to DSBs and subsequent remodelling by NuA4, SWR1, and INO80 (Javaheri et al., 2006).

The INO80 complex (INO80.com) displays nucleosome-stimulated ATPase activity and ATP-dependent chromatin remodelling activity *in vitro* (Jonsson et al., 2001; Zophonias et al., 2004). Moreover, INO80 has also been shown to relocate nucleosomes to more central positions on short DNA fragments (Jin et al., 2005). Potentially both these properties may be employed by the INO80.com to produce single stranded regions during repair of DNA breaks *in vivo* (van Attikum et al., 2004). Moreover, INO80 has been found to be recruited to sites of DNA damage. It has been shown that H2A phosphorylation allows binding of NuA4, SWR1, and INO80 complexes; perhaps exposing H3 diMe-K79 (Javaheri et al., 2006). In mammals, the NuA4 and Ino80 complexes are practically combined in the form of the Tip60 complex (Morrison et al., 2005).

1.3.5.4.3. EP400

The EP400 subfamily archetype, E1A-binding protein p400, was first identified based on its role in the regulation of E1A-activated genes (Samuelson et al., Chan et al., 2005; Fuchs et al., 2001). The human EP400 protein is closely related to the human homolog of Swr1, SRCAP (Snf2p-related CBP activating protein) with 50% identity suggesting a close functional relationship (Flaus et al., 2006). However, HMM (Hidden Markov Models) profiles clearly distinguish EP400 from SWR1 members, and show that EP400 members are restricted to vertebrates whereas SWR1 subfamily members are found in almost all eukaryotes (Flaus et al., 2006). Although most vertebrate genomes contain a gene each for a SWR1 and EP400 member, some have only one of the pair (Flaus et al., 2006).

EP400 has also been found to interact with many proteins also found to interact with SRCAP. For example, EP400 has been shown to interact strongly with RuvB-like

helicases TAP54α/ß in the TRRAP/TIP60 histone acetyl transferase complex (Fuchs et al., 2001). Complexes of potentially overlapping composition exist involving human EP400 and SRCAP including the NCoR-1 histone deacetylase (Underhill et al., 2000), TRRAP/TIP60 histone acetylase (Cai et al., 2005; Fuchs et al., 2001; Cai et al., 2003) and DMAP1 complex (Doyon and Cote, 2004; Doyon et al., 2004).

Outside of the ATPase domains, the two proteins differ, Human EP400 contains SANT domains, whereas human SRCAP instead contains an AT hook (Eissenberg et al., 2005; (Johnston et al., 1999). This has led to some confusion; with human EP400 being referred to as hDomino although *D.melanogaster* Domino has higher similarity to human SRCAP (Fuchs et al., 2001) and SRCAP can complement Domino mutants (Eissenberg et al., 2005).

It has been suggested that in *D.melanogaster* alternative splice isoforms DominoA and DominoB are functional homologues of EP400 and SRCAP, respectively and that *S.cerevisiae* Eaf1p is a functional homolog of human EP400 although it lacks both Snf2-related helicase-like and extended proline-rich regions (Monroy et al., 2003; Johnston et al., 1999). The human member SRCAP acts as a transcriptional co-activator of steroid hormone dependent genes and has recently been shown to be a component of a human TRRAP/TIP60 complex (Cai et al., 2005) and other complexes. It also interacts with several coactivators including CBP (Johnston et al., 1999; Monroy et al., 2003). SRCAP can rescue *D.melanogaster* domino mutants (Eissenberg et al., 2005), implying functional homology.

The EP400 homolog in the *A.thaliana* PIE1 (Photoperiod-Independent Early flowering) is involved in vernalization regulation through a pathway intimately linked with histone lysine methylation events (Noh and Amasino, 2003; He and Amasino, 2005).

1.3.5.4.4. Etl1

Etl1 (Enhancer Trap Locus 1) derives its name from the mouse Etl1 which was identified in an expression screen for loci having the ability to drive the expression of Beta-galactosidase reporter gene (Soininen et al., 1992). Etl1 is expressed widely with especially high levels present in the central nervous system and epithelial cells (Schoor et al., 1993 and Schoor et al., 1999). Etl1 is non essential, although deletion causes a variety of significant developmental phenotypes such as skeletal dysplasia, growth retardation, and impaired infertility (Schoor et al., 1993; Schoor et al., 1999). The amino acid sequence of the Etl1 protein deduced from the cDNA revealed strong homology to other Snf2 family proteins (Ouspenski et al., 1999). It is now clear that Etl1 is most closely related to members of the Swr-1 grouping (Flaus et al., 2006). The expression pattern during embryogenesis suggests that Etl1 protein might function in pathways during mouse development (Ouspenski et al., 1999).

The human Helicase 1 (hHel1), now designated SMARCAD1 (SWI/SNF-related, Matrix-associated, Actin-dependent Regulator of Chromatin, containing DEAD/H box 1) is the human homolog of mouse Etl1 (Fig. 1.3) (Korn et al., 1992). SMARCAD1 is a DEAD/H box-containing protein that has seven highly conserved sequence regions that allows it to be placed in the SNF2 family of the helicase superfamily (Korn et al., 1992). Moreover, SMARCAD1 contains two DEAD/H box motifs, a property reported to be shared by Swr-1 grouping and not any other SNF2 family members (Korn et al., 1992). In addition, SMARCAD1 has a putative nuclear localization signal and several regions that may mediate protein-protein interactions (Korn et al., 1992). Recently, it has been shown by high-resolution genome tiling microarrays in chromatin immunoprecipitation that the binding sites of SMARCAD1

in the vicinity of the transcriptional start site of 69 candidate target genes, one of them is TRIM28, a recently highlighted transcriptional regulator in the cancer field. From these findings (Okazaki et al., 2008).

Expression analysis indicates that similarly to Etl1, SMARCAD1 transcripts are ubiquitous, with particularly high levels in endocrine tissue (Korn et al., 1992; Adra et al., 2000). The gene for hHel1 has been mapped to the chromosome 4q22-q23m which is rich in break points and deletion mutations involved in several human diseases, notably soft tissue leiomyosarcoma, hepatocellular carcinoma, and hematologic malignancies (Korn et al., 1992; Adra et al., 2000; Okazaki et al., 2008).

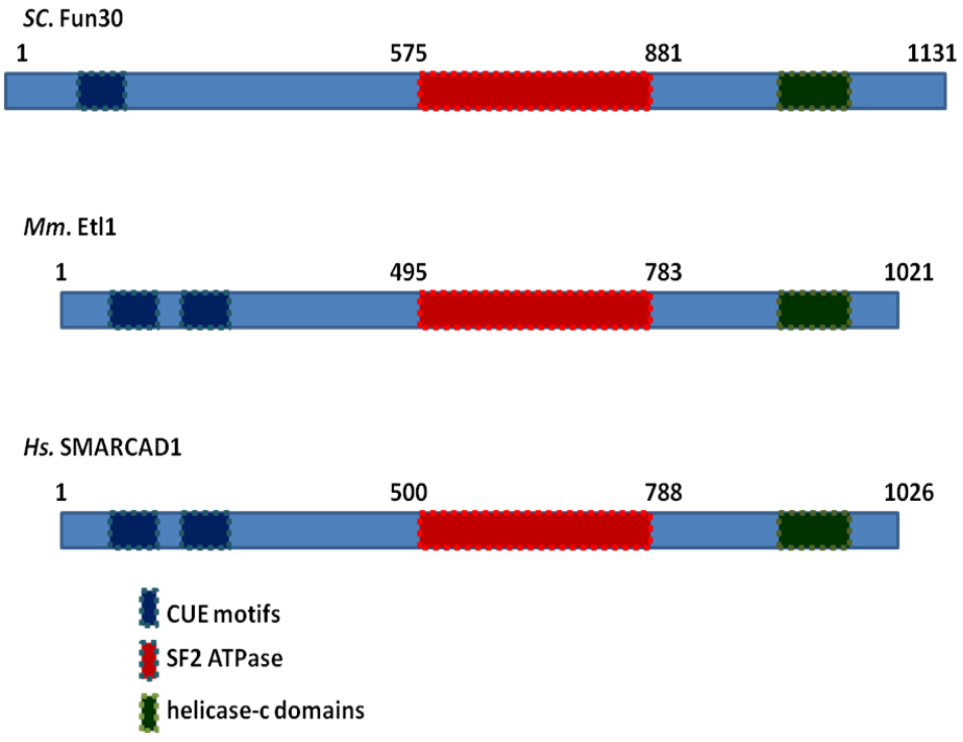

Figure 1.3: Schematic model of sequence homology between Saccharomyces cerevisiae (Sc) Fun30, and Human / mouse (Hs, Mm) SMARCAD1/ETL1. Relative positions of putative CUE motifs (blue), SF2 ATPase (red) and helicase-c domains (green). The amino acid positions for the SNF2_N domains are indicated. Modefied from (Neves-Costa et al., 2009).

1.3.5.4.5. Fun30

The yeast Fun30 (Function Unknown Now 30) protein is encoded by a gene located on chromosome I of the *S. cerevisiae* and is 128 KDa in size. Fun30, belongs to the Snf2 family and is a member of the Etl1 (Enhancer Trap locus 1) subfamily (Flaus et al., 2006). A more detailed description of Fun30 will be discussed later on Chapter 3.

1.4. Roles for chromatin in genetic processes

The involvement of chromatin in the regulation of genomic functions is well established. Changes in chromatin structure are the basis of many regulatory processes; in particular, transcription, replication and DNA repair.

1.4.1. Chromatin structure and Transcription

The packaging of genes as chromatin impedes transcription. This may involve the occlusion of the binding sites of transcription factors and other components of the transcriptional machinery. However, it is worthy of mentioning that some transcription factors are capable of recognizing their sites in nucleosomal DNA (Cavalar et al, 2003). In addition there is some evidence that DNA sequences at key regulatory elements may act to directly exclude nucleosomes (Vitolo et al. 2000; Protacio et al. 2000). As a result it is possible that pioneering transcription factors may bind and act to recruit proteins that act to reconfigure chromatin structure. These include enzymes that act to post translationally modify chromatin components and ATP-dependent chromatin remodelling enzymes. There may be some interplay between these processes. Chromatin-modifying/remodelling complexes facilitate specific steps in transcription. For example the ATP-dependent remodelling complexes can increase binding by gene-specific activators (Cote et al. 1994 and

Imbalzano et al. 1994). Whilst at specific genes there often is a defined order in which different chromatin modifying complexes are recruited, this can vary from gene to gene (Narlikar et al., 2002; Metivier et al. 2003; Nagaich et al. 2004; Ikeda et al. 1999). Ultimately the result of these chromatin rearrangements is the provision of a platform that allows appropriate recruitment of basal transcription factors and RNA polymerase.

1.4.2. DNA replication and chromatin

Eukaryotic cells duplicate their DNA following a chronological programme directed at ensuring that entire genomes are replicated one and only once per cell cycle. DNA replication occurs in the S-phase of the cell cycle, in yeast there are defined origins known as Autonomously Replicating Sequence (ARS) elements. Some replication events take place early and others occurring towards the end of the S-phase period (Donaldson, 2005). The bulk of the cell cycle knowledge has been achieved by studying the budding yeast Saccharomyces cerevisiae. The minichromosome maintenance (or MCM) proteins were first identified by genetic screens in yeast and subsequently found to be the central players in the initiation of DNA replication. More recent studies have shown that MCM proteins also function in replication elongation and many aspects of genome stability, probably as a DNA helicase (Forsburg, 2004). It has been indicated that chromatin remodelling factors play a vital role in the regulation of replication origin. For instance, Trachtulcova and colleagues found that ISW2 is required for premeiotic DNA replication (Trachtulcova et al. 2000).

To coordinate DNA-replication and -repair processes in the context of chromatin is a challenge, both in terms of accessibility and maintenance of chromatin organization. To meet the challenge of maintenance, cells have evolved efficient nucleosome-

assembly pathways and chromatin-maturation mechanisms that reproduce chromatin organization in the wake of DNA replication (Groth et al., 2007). Many transcription factors have been shown to activate DNA replication. It has been suggested that transcription factors bind to auxiliary sequences adjacent to replication origins and recruit chromatin remodelling factors to create either nucleosome-free regions or regions of specifically spaced nucleosomes (Melendy and Li, 2001). This results in activation of the nearby origin, by allowing more accessibility to replication factors.

Some studies have provided data indicating that direct interactions may exist between chromatin remodelling factors and two cellular replication factors, the Origin Recognition Complex (ORC) and Proliferating Cell Nuclear Antigen (PCNA) (Melendy and Li, 2001). It has been reported that the SWI/SNF complex stimulates replication in various cases, either directly or indirectly (reviewed in Melendy and Li, 2001). Moreover, ORC1 was found to be associated with a histone acetyltransferase (HAT), HBO1 (Melendy and Li, 2001). However, since these replication factors are also involved in other nuclear processes, such as transcriptional silencing and DNA repair, respectively, further study is necessary to establish whether these direct interactions are also important for DNA replication.

The first comprehensive study of the DNA replication in budding yeast *S. cerevisiae* showed that there is no correlation between replication time and transcriptional status (Raghuraman et al., 2001), which might be because the yeast genome is mostly active. On the other hand, a subsequent analysis in *Drosophila* proposed that early-replicating genes are more liable to be transcribed than late-replicating genes (Schubeler et al., 2002). These findings raised the question of whether the mechanisms that underline the replication time of active and inactive chromatin vary

between yeast and the rest of higher eukaryotes. Several subsequent studies provided new insights into this dilemma. The first complete-genome study of replication timing in human cells has found a clear relationship between replication time and other features that correlated with gene expression such as gene density and GC content of the DNA (Woodfine et al, 2004). Throughout the process of DNA replication, DNA polymerases and the enzymes involved in the replisome must overcome many obstacles that impede the replication fork progression. Nucleosomes must be disassembled ahead of the replication fork and reassembled behind it (Linger and Tyler, 2007).

1.4.3. DNA damage and repair mechanisms

The DNA damage response (DDR) has evolved to overcome the destructive effects of DNA damage, which occurs continuously by both intrinsic and extrinsic sources. The DDR was defined as the harmonized interaction between DNA repair pathways, which work to reverse DNA damage, and checkpoint pathways, which block progression through the cell cycle while repair occurs (Alderton, 2008; Nyberg et al., 2002). Transient DNA damage occurs normally during DNA replication, transcription, and recombination and can arise spontaneously for example as a result of irradiation. These forms of DNA damage all have the potential to prevent the inheritance of genetic information from one generation to another. To help counter the problems associated with DNA damage a series of checkpoints arrest progression through the cell cycle until DNA replication is complete. These include: G1/S (G1) checkpoint, intra-S phase checkpoint, and G2/M checkpoint (Russell, 1998).

Central to this network are protein kinases known as Tel1/Mec1 in budding yeast and Tel1/Rad3 in fission yeast (McGowan and Russell, 2004). These kinases sense DNA damage and phosphorylate a number of target proteins that regulate cell cycle

progression and DNA repair pathways. Mec1 is a member of the conserved family of phosphatidylinositol 3-kinases (PI3-kinases) and has homology to the fission yeast Rad3 and human ATM and ATR (Gunjan and Verreault, 2003). Mec1 phosphorylates several targets including Rpa1 and Rpa2 subunits of RPA, the Rad24 subunit of the clamp loader, the Ddc1 and Mec3 subunits of the clamp. The effecter kinase Rad53 is also efficiently phosphorylated by the activated form of Mec1 (Gunjan and Verreault, 2003).

Mammalian cells utilize five major DNA repair pathways: Homologous Recombination Repair (HRR); Nonhomologous End Joining (NHEJ); Nucleotide Excision Repair (NER); Base Excision Repair (BER) and Mismatch repair (MMR) (Ataian and Krebs, 2006). MMR, NER and BER pathways are excision repair processes that depend upon complementary DNA strands to direct their replacement of excised base(s). The MMR pathway repairs mismatches and loops generated by insertions or deletions. The BER pathway recognizes and removes incorrect or damaged bases using a family of DNA N-glycosylases that result in the formation of an apurinic/apyrimidinic (AP) site. There are two major types of BER repair mechanisms, one involves DNA polymerase beta-dependent single nucleotide repair and the other, repair of long-patches (2-10 nucleotides) and this requires PCNA. Whereas, BER processes are dependent upon specific N-glycosylases to recognize mismatches or damage, the NER pathway recognizes abnormal structures using proteins such as DDB1, XPE, XPC or HR23B. Defect in NER have been associated with Xeroderma pigmentosum (XP), Cockayne's syndrome (CS) and trichothiodystrophy (TTD).

Repair of double strand DNA breaks (DSB) is mediated by homologous recombination (HRR) or nonhomologous end joining (NHEJ) pathways. HRR

employs one of two related but distinct mechanisms. Synthesis-dependent strand annealing (SDSA) requires *RAD51, RAD52, RAD54, RAD55, RAD57* and *RAD59* and requires that a single DNA strand find its complementary sequence within a double-stranded DNA. Single-strand annealing (SSA) only requires the association of two complementary strands and RAD 52 and 59. NHEJ is homologous DNA template-independent and facilitates direct modification and ligation of two DNA ends present in DSB (Ataian and Krebs, 2006).

1.4.3.1. Chromatin alterations and DNA repair

DNA repair occurs within the context of chromatin. As a result it is not surprising that chromatin alterations are associated with DNA repair. For example, the histone variant H2AX is phosphorylated within the vicinity of sites of DNA damage and forms discrete, microscopically visible foci (Lisby and Rothstein, 2004; Lisby et al., 2004; Pinto and Flaus, 2010). In yeast there is no histone H2AX variant, but the major form of H2A is phosphorylated in a similar way. H2AX, which is modified by upstream checkpoint kinases, and it is thought that the phosphorylated protein acts to recruit downstream DNA repair proteins (Shroff et al., 2004; Lydall and Whitehall, 2005) such as the HAT complex NuA4 as well as the ATP-dependent remodeler INO80 to sites of DNA damage (Downs et al. 2004; Morrison et al. 2004). The association of NuA4 with chromatin neighboring a DSB is mediated by its Arp4 subunit and appears to be direct as Arp4 interacts with a H2A phospo-S129 peptide (Downs et al., 2004). Arp4 is one of a number of actin-related proteins that are found in various chromatin remodelling complexes and indeed Arp4 is not only a component of NuA4, but is also an integral subunit of two other multisubunit ATP-dependent remodelling complexes INO80 and SWR1 (Shen et al., 2003). INO80 is recruited to HO endonuclease-induced double strand breaks (van Attikum et al.,

2004) and it has been proposed that its interaction with phosphor- H2A is dependent on a subunit called Nhp10 (van Attikum et al., 2004). INO80 appears to have roles in both non-homologous end joining and homologous recombination repair pathways (Shroff et al., 2004). Notably, the loss of INO80 function leads to defects in the generation of 3′ ssDNA overhangs (van Attikum et al., 2004). This finding suggests that one function of INO80 is to remodel chromatin in order to allow efficient DSB processing by nucleases (Shroff et al., 2004).

SWR1 also has been shown to be recruited to DSBs. It has recently been proposed that INO80 and SWR1 function antagonistically at chromatin surrounding a DSB, and they regulate the incorporation of different histone H2A variants that can either promote or block cell cycle checkpoint adaptation (Papamichos-Chronakis et al., 2006). Interestingly, a new study demonstrated that Ino80 has a histone-exchange activity in which the enzyme can replace nucleosomal H2A.Z/H2B with free H2A/H2B dimers (Papamichos-Chronakis et al., 2011).

1.4.3.2. Cell cycle regulation and DNA damage

Cell cycle events must be highly regulated in a temporal manner. Genetic and molecular studies in diverse biological systems have resulted in identification and characterization of the cell cycle machinery. The cell cycle is regulated by cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CDKIs) and is divided into 4 distinct phases (G1: Gap 1, S: DNA synthesis, G2: Gap 2, M: Mitosis) (Grana and Reddy, 1995; Johnson and Walker, 1999). G0 represents exit from the cell cycle. The cell cycle is driven by CDKs, which are positively and negatively regulated by cyclins and CDKIs, respectively (Grana and Reddy, 1995). The cell cycle is controlled by both internal and external signals that either stimulate or inhibit progress through the cycle (Murnane, 1995).

Transient DNA damage occurs normally during DNA replication, transcription, and recombination and can arise spontaneously for example as a result of irradiation. These forms of DNA damage all have the potential to prevent the inheritance of genetic information from one generation to another. To help counter the problems associated with DNA damage all organisms have developed a series of responses. These include 1) activation of cell cycle checkpoints, and 2) initiation of accurate DNA repair (Murnane, 1995).There are three major checkpoints where progression through the cell cycle can be regulated. According to the cell cycle stages, DNA damage checkpoints are classified into at least 3 checkpoints: G1/S (G1) checkpoint, intra-S phase checkpoint, and G2/M checkpoint (Russell, 1998).

DNA replication and chromosome distribution are crucial events in the cell cycle control. Cells must accurately copy their chromosomes, and through the process of mitosis, segregate them to daughter cells. The checkpoints act as surveillance and quality control mechanism of the genome to maintain genomic integrity. Checkpoint failure often causes mutations and genomic arrangements resulting in genetic instability. Studies on many different species have uncovered a network of proteins that form the DNA damage checkpoints. Central to this network are protein kinases known as Tel1/Mec1 in budding yeast and Tel1/Rad3 in fission yeast (McGowan and Russell, 2004). These kinases sense DNA damages and phosphorylate number of proteins that regulate cell cycle progression and DNA repair pathways.

There are two main essential protein kinases, Mec1 and Tel1, which play multiple roles in the DNA damage response in *Saccharomyces cerevisiae*. *MEC1* and a structurally related gene, *TEL1*, have overlapping functions in response to DNA

damage and replication blocks that can be substituted for by overproduction of *RAD53*, the yeast homolog of *CHK2* (Sanchez al., 1996). Mec1 and Rad53 act to prevent DNA replication following DNA damage (Gunjan and Verreault, 2003). Thus, the DNA damage will lead to a sudden decrease in DNA synthesis (Paulovich and Hartwell, 1995). Both *MEC1* and *TEL1* have been found to control phosphorylation of Rad53p in response to DNA damage. These observations indicate that Rad53 is a signal transducer in the DNA damage and replication checkpoint pathways and functions downstream of two members of the ATM lipid kinase family. Because several members of this pathway are conserved among eukaryotes, it is likely that a Rad53-related kinase will function downstream of the human ATM gene product and play an important role in the mammalian response to DNA damage (Sanchez wt al., 1996).

1.5. Aims and objectives

The yeast Fun30 protein can be identified as a Snf2 family member based upon sequence homology. To gain insight into the mechanism of action of this chromatin remodelling protein, it will be of value to determine how its activities are regulated, how it is targeted to specific genes, and how it interacts with other chromatin modifying enzymes and other regulatory proteins.

Our overall objective is to better understand the mechanisms by which the Fun30 protein is involved in chromatin remodelling and transcription regulation.

The specific aims of this proposal are:

I) Purification and identification of the subunits of the Fun30 protein complex.

II) Investigating the mechanism of action of Fun30 using biochemical techniques.

III) To investigate the functions of Fun30 based on analysis of phenotypes and genomic approaches.

To achieve this we will use a combination of genetic, biochemical and molecular approaches to study the mechanism of action of the putative Fun30 chromatin remodelling complex. Results obtained from these studies should be valuable to the scientific community regarding the mechanisms of transcription regulation, as well as to the medical community since mutations in genes encoding subunits of human chromatin-remodelling complexes are associated with cancer and other diseases.

Ch. II. Materials and Methods

2.1. Purification and characterization of SWI/SNF, RSC and Fun30

The SWI/SNF and RSC complexes as well as Fun30 were purified from yeast whole cell extract by Tandem Affinity Purification (TAP) over two affinity columns (Puig et al., 2001) see Fig. 2.1. The Snf6TAP-tag strain used for the purification of the SWI/SNF was generated by M. Carroza, for RSC the strain BCY211 was kindly supplied by Brad Cairns and the Fun30 TAP-tag strain was purchased from Euroscarf (see Table.2.1. for full yeast strain list). The yeast were grown at 30 ^{o}C in 6 *x* 2 L flasks, with each flask containing 1 L of 3 *X* YPD medium (3% bactoyeast extract, 3% bactopeptone, 6% glucose), with shaking at 200 rpm until the optical density at 600 nm reached 2.5-3. Cells were pelleted at 4 ^{o}C in a JS 4.2 rotor at 4000 rpm for 20 minutes. The cell pellet was resuspended in 15 ml of remodeler buffer (20 mM HEPES pH 7.4, 350 mM sodium chloride, 10% glycerol, 0.1% Tween-20, 2 µM E-64, 1 mM AEBSF (4-(2-aminoethyl)benzenesulphonyl fluoride), 1 µM pepstatin, 2.6 mM aprotinin) and frozen by drop-wise addition into liquid nitrogen. The cells were lysed by using of either BSP-Bead Beater (HamiltonBeach Commercial, UK) or the Grabdpix2008 (Restsch, Germany). Typically, blending achieved 70-90 % lysis, with the proportion of lysis examined with a light microscope. The lysed sample was stored overnight at -20 ^{o}C in an open container.

All purification steps were carried out at 4 ^{o}C. The lysed sample was thawed and centrifuged at 31000 g in a JA-25.50 rotor for 15 minutes. The supernatant was recovered and ultracentrifuged in pre-cooled Beckman Coulter Ti45 rotor at 40000 g for 45 minutes. The central layer of the lysate was added to 400 µl of IgG sepharose 6 Fast Flow beads (GE Healthcare) which binds to protein A of the TAP-tag. The

sample was left to rotate for 2 hours, before loading onto a 10 ml Poly-Prep chromatography column (BioRad). The column was washed with 30 ml of remodeler buffer and 10 ml of TEV cleavage buffer (remodeler buffer with 1 mM DTT). The base of the column was closed and 2 ml of TEV cleavage buffer was added with 300 Units of TEV (Tobacco Etch Virus) protease, the column was rotated overnight. The flow-through was collected and the column washed with 2 ml of TEV cleavage buffer.

To a new 10 ml Poly-Prep column, 400 µl of calmodulin affinity resin (Stratagene) which will bind to the calmodulin binding domain in the tagged protein was added. After that the calmodulin resin was washed with 10 ml of calmodulin binding buffer (remodeler buffer with 2 mM calcium chloride). The column was closed and the TEV eluate added with 2 mM calcium chloride, followed by rotation for 2 hours. The column was washed with 20 ml of calmodulin binding buffer. The sample was eluted with 500 µl of calmodulin elution buffer (remodeler buffer with 10 mM EGTA, with the column left to stand 20 minutes between each 0.25 ml fraction.

The eluate was concentrated with a YM-50 Microcon spin concentrator (Millipore) at 14000 g to a 100 µl volume and dialyzed overnight against remodeler storage buffer (remodeler buffer without protease inhibitors) using a microdialysis apparatus and 12000-14000 Da MWCO dialysis membrane (SpectraPor 2.1). The dialyzed sample was aliquoted in 10 µl volumes and frozen on dry ice before storage at -80 ^{o}C. To quantitate the yield, an aliquot was run on a NuPAGE 4-12 % Bis-Tris polyacrylamide gel. Purification was monitored by western blotting, using anti-TAP antibody (Open Biosystems, Huntsville, AL, U.S.A) as well as silver staining. Equivalent quantities of the enzymes were used in assays after normalization for the

amounts of purified protein present monitored by western blotting using an anti TAP antibody.

For the low stringent purification of Fun30, the TAP technique was used with the exception that the washing and elution steps were carried in 80mM NaCl solutions.

For the co-immunoprecipitation experiment, the TAP-tagged strain was transformed with a GAL HA tagged Fun30 expression plasmid (Open Biosystems, Huntsville, AL, USA). Following induction, total cell protein was purified from 500 ml minimal media culture inoculated with either the single or the double-tagged yeast strains. The protein concentration was measured by Bradford assay, and 60 µg total protein was incubated with either IgG sepharose beads (GE Healthcare, Sweden) or Protein G Plus Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) coupled to anti-hemagglutinin (HA) high-affinity rat monoclonal antibody 3F10 (Roche) in a pulldown buffer (50 mM HEPES [pH 7.5], 1 mM EDTA, 150 mM NaCl, 10% glycerol, 0.1 % Tween 20, 0.5 mM DTT, 1 mM PMSF, 2 µg/ml leupeptin, and 2 µg/ml pepstatin A for at least 2 hr at 4°C while mixing on a rotating wheel. The supernatants were collected, and the beads were then washed with pulldown buffer three times and left as a 50% slurry after the final wash. Equal fractions of both supernatants and beads were loaded on a 10 % SDS-PAGE gel, and the presence of Fun30 was detected by immunoblotting.

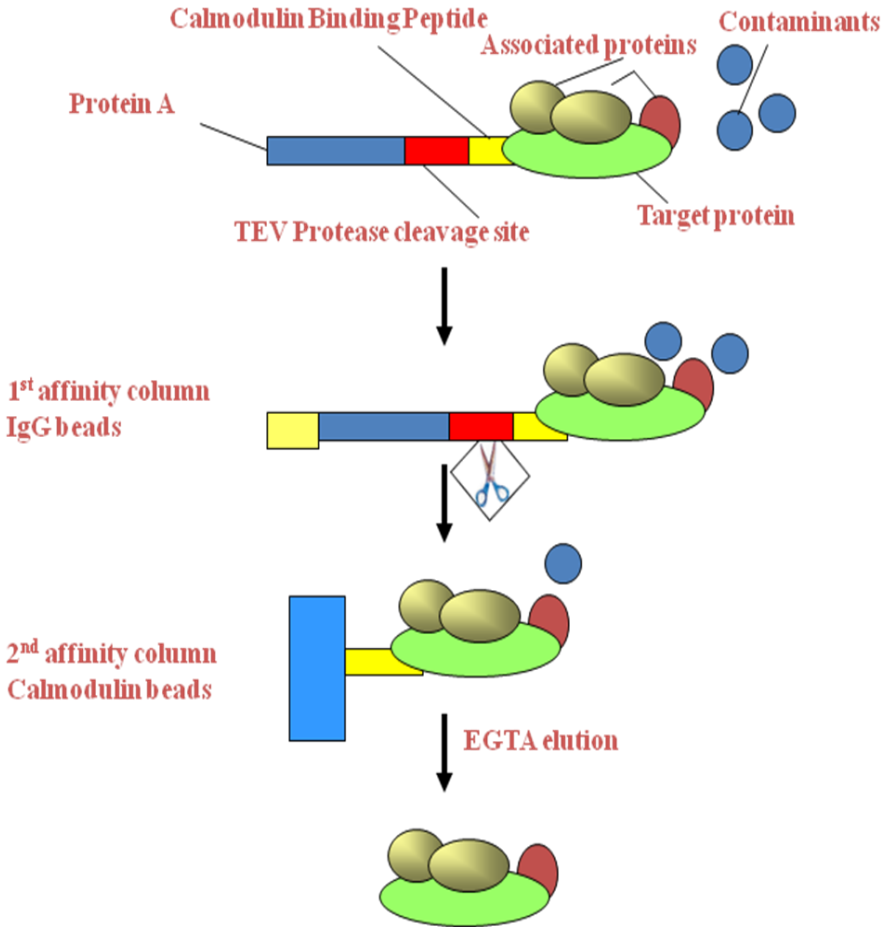

Figure 2.1. Representation of the TAP purification technique. The TAP purification method involves the fusion of the TAP tag (protein A, TEV clevag site, and CBP) to the target protein of interest and the introduction of the construct to the cognate host cell or organism. The fusion protein and associated components are recovered from extracts by affinity selection on an IgG-matrix which interacts with protein A of the tag. After washing, the TEV protease is added to release the bound material. The eluate is incubated with calmodulin-coated beads in the presence of calcium which intercat with the CBP. This second affinity step is required to remove the TEV protease as well as traces of contaminants remaining after the first affinity selection. After washing, the bound material is released with EGTA.

2.2. Expression and purification of recombinant His-tagged Fun30

The Fun30 gene was amplified from yeast genomic DNA and subsequently subcloned into the pGEX-6P1 expression plasmid (pGF30). pGF30 was then engineered to contain an in-frame hexa-histidine tag (SSHHHHHH; 6xHis) at the C-terminus of the Fun30 gene (pGF306H). Double-tagged Fun30 (N-terminal GST-tag, C-terminal 6XHis; GST-Fun30-6XHis) was expressed from pGF306H in the Rosetta2 *E. coli* strain (Novagen) at 20 °C overnight by induction with 0.4 mM IPTG. Cells were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris pH 7.5, 350 mM NaCl, 0.05 % β-mercaptoethanol) containing protease inhibitors (0.2 mM AEBSF, 2 µM E64, 2.6 mM aprotinin, 1 µM pepstatin) with or without 0.1 % Tween-20 as indicated. Cells were lysed by freeze/thawing in liquid nitrogen and sonication. The soluble fraction was extracted by centrifugation at 35000 *g* at 4 °C for 30 min. GST-Fun30-6xHis was then purified in batch over HisPur cobalt resin, washed in lysis buffer, and eluted in lysis buffer containing 250 mM imidazole. The HisPur eluate was then applied to SuperGlu glutathione resin (Generon) and washed in lysis buffer then Prescission protease buffer (20 mM Tris pH 7.5, 350 mM NaCl, 1 mM DTT, 1 mM EDTA) with or without the inclusion of 0.1% Tween-20. Fun30-6XHis was then released from the GST-tag by cleavage overnight with Prescission protease and subsequently eluted in Prescission protease buffer. Fun30-6XHis was then concentrated in 50 KDa MWCO centrifugal concentrators and dialyzed against 20 mM Tris pH 7.5, 350 mM NaCl, 10% Glycerol, 1 mM DTT and stored at -80 C.

2.3. Octamer, tetramer and dimer assembly and purification

Octamer, tetramer and dimer assemblies were carried out according to a published protocol (Luger et al., 1999) with minor alterations. For each assembly, lyophilised

histones were dissolved in 500 µl of unfolding buffer (7 M guanidinium hydrochloride, 20 mM sodium acetate pH 5.2, 10 mM DTT). The absorbances at 276 nm were measured and the histones were mixed in an equimolar ratio. Samples were dialyzed at 4 ^{o}C using 6000-8000 Da MWCO membrane (SpectraPor 2.1, USA) against 3 *X* 1 L of refolding buffer A (2 M sodium chloride, 10 mM Tris pH 7.5, 1 mM EDTA, 5 mM β-ME), with at least 3 hours stirring per step. Samples were centrifuged at 20817 g for 10 minutes in a 4 ^{o}C Eppendorf benchtop centrifuge, before concentration with a YM-10 Centricon spin concentrator (Millipore, USA) to 200-300 µl. Samples were loaded onto a Superdex 200 10/300 GL (GE Healthcare) gel filtration column. The column was run on an Äkta purifier system (GE Healthcare) with refolding buffer (with 200 µM DTT instead of β-ME) at 0.3 ml/min and 333 µl fractions were collected between 11-20 ml. Typically, octamers eluted at 13.3 ml, tetramers 14.2 ml and dimers at 16.3 ml. Fractions under the symmetrical part of the peak were pooled, avoiding the asymmetric tail on the back half of the peak as this contains substoichiometric assemblies. The pooled fractions were concentrated with a YM-30 Microcon spin concentrator (Millipore) to achieve a concentration of 20-30 µM for octamer and tetramer assemblies and 80-100 µM for dimer assemblies. Samples were stored at 4 ^{o}C for up to 6 months for octamers and up to 3 months for dimers and tetramers.

2.4. Generation of fluorescently labeled dimers

Dimers were fluorescently labeled by coupling a cy5 mono maleimide dye (GE Healthcare) at a cysteine inserted at H2A T10 (see section 8.1 for more details). The dimers were prepared as described in Section 2.3, with the following changes. Unfolding buffer contained 50 mM DTT. After the histones were mixed the sample was heated at 50 ^{o}C for 20 minutes before dialysis to further reduce the cysteine

residue and ensures the complete unfolding. Refolding buffer B for dialysis and gel filtration contained 10 mM sodium acetate pH 5.2 and no β-ME or DTT. The cy5 dye was dissolved in a small volume of DMSO (dimethyl sulphoxide) and added at an equimolar ratio to the purified dimers. The reaction was adjusted to 10 mM HEPES buffer pH 7.0 and incubated in the dark at room temperature for 3 hours. The labeled dimers were dialyzed at 4 ^{o}C using 6000-8000 Da MWCO dialysis membrane against 3 *X* 1 L refolding buffer, with stirring of each step for at least 3 hours. The volume after dialysis was used to estimate the dimer concentration.

2.5. Preparative PCR

PCR (polymerase chain reaction) was used to generate sufficient quantities of defined DNA fragments for nucleosome assembly. All fragments were amplified from 2 source sequence plasmids; the artificial 601.3 positioning sequence in a pGEM-3Z plasmid (Promega, USA) kindly provided by the Widom lab (Anderson et al., 2002) and the MMTV nucA (nucleosome A) sequence in a pDONOR201 plasmid (Invitrogen). The nomenclature adopted to define the DNA fragments is *aBc*. *a* and *c* represent the upstream and downstream bp extensions on either side of nucleosome core, respectively. *B* denotes the sequence origin, with W and A representing the 601.3 and MMTV nucA sequence, respectively. 5 ml PCR reactions were prepared on ice and contained 1 *X* NH_4 PCR buffer (16 mM ammonium sulphate, 0.01 % Tween-20, 67 mM Tris pH 8.8), 0.4 ng/µl template plasmid, 1 µM of each primer, 2 mM magnesium chloride, 120 µM dNTPs (Bioline, USA) and 20 µl DNA *Taq* polymerase purified by Tom Owen-Hughes. 5% (v/v) DMSO was added for amplification of fragments from the 601.3 template. The reaction was aliquoted in 50 µl volumes into a ThermoFast 96 well low profile plate (ABgene, UK) and incubated in a Eppendorf mastercycle at 94 ^{o}C for 2 minutes, followed by

30 cycles of 94 ^{o}C for 30 seconds, 50 ^{o}C for 30 seconds and 72 ^{o}C for 1 minute, with a final incubation at 72 ^{o}C for 5 minutes.

The PCR samples were pooled in a 50 ml conical tube and ethanol precipitated by addition of 0.1 *X* 3 M sodium acetate pH 5.2 and 3 *X* 100% ethanol. This was briefly vortexed before centrifugation at 5200 g for 20 minutes in a Heraeus Megafuge 1.0 centrifuge (Thermos Fischer Scientific, USA). The pellet was air dried for 5 minutes and resuspended in 500 µl of ion exchange buffer A (10 mM Tris pH 7.5, 0.1 mM EDTA). The sample was centrifuged for 10 minutes at 20817 g in a benchtop centrifuge before purification by anion exchange chromatography. After that the concentration of DNA was measured by spectrometry and usually the 5 ml PCR reaction result in a yield ranges between 10-20 µg.

2.6. Purification of PCR products by ion exchange chromatography

Anion exchange chromatography was performed on a BioCAD Sprint (Applied Biosystems, USA) using a 1.8 ml SOURCE 15Q column (GE Healthcare). The DNA fragments were separated from primers and dNTPS by 2 stages of gradient elution with ion exchange buffer A and ion exchange buffer B (2 M sodium chloride, 10 mM Tris pH 7.5, 0.1 mM EDTA). The column was run at a flow rate of 2 ml/min with an initial 3 ml wash of ion exchange buffer A, followed by the first gradient of a 0-26% linear increase of ion exchange buffer B over 5 ml and then the second gradient of a 26-40% ion exchange buffer B over 13 ml. The appropriate fractions were pooled and ethanol precipitated as described in Section 2.1.4. The pellet was resuspended in a small volume of ion exchange buffer A, the 260 nm absorbance measured to determine the concentration, before storage at -20 ^{o}C.

2.7. Chromatin assembly

Chromatin was assembled onto the GUB (Gal USF pBend) template from purified HeLa oligonucleosomes by dilution from high salt as described previously (Hassan et al., 2006). Recombinant histone octamers were assembled from individual *Xenopus laevis* histones expressed in bacteria (Luger et al., 1997). Octamers were reconstituted onto nucleosomes using DNA synthesised by PCR from the MMTV nucleosome A (NucA) (Flaus et al., 2003). Reconstitutions were performed at 1 µM concentration and pH 7.5 by stepwise dialysis from 2 M NaCl or KCl to 0.85 M, 0.65 M, 0.5 M and finally 0 M. H2B was fluorescently labelled by attachment of mono maleimide dye (GE Healthcare) as described previously (Bruno et al., 2003). It worth mentioning here that both H2A/H2B could be coupled with fluorescent dye. After that tetrasomes for dimer transfer were assembled at a substoichoimetric ratio of tetramer (0.8 fold) to DNA, to promote mono-tetrasome assembly.

All recombinant histone assemblies were performed by dialysis at 4 ^{o}C against progressively lower salt solutions using a specifically created microdialysis apparatus and 6000-8000 Da MWCO dialysis membrane. Samples were dialyzed against 8 ml buffer A (0.85 M sodium chloride, 10 mM Tris pH 7.5), buffer B (0.65 M sodium chloride, 10 mM Tris pH 7.5), buffer C (0.5 M sodium chloride, 10 mM Tris pH 7.5) and buffer D (0.1 M sodium chloride, 10 mM Tris pH 7.5) with each step stirred for at least 1.5 hours and the last dialysis performed overnight. Nucleosomes were stored for up to a month at 4 ^{o}C.

2.8. Immobilized template binding assay

A 2.5 kb fragment excised from plasmid pG5E4-5S that contains a dinucleosome length G5E4 fragment flanked on both sides by five 5S sequences was prepared as described (Hassan et al., 2002). Following reconstitution by step dilution as indicated

the arrays were coupled to streptavidin Dynabeads (Dynal). 10 nM of either SWI/SNF or Fun30 was added to 200 ng of the above template in 20 µl binding buffer and incubated for 1 hour at 30 °C. The templates were then concentrated using a magnet, the supernatant was removed, and the beads were washed twice. The presence of SWI/SNF and Fun30 was determined by Western blotting using the anti-TAP antibody.

2.9. Quantitative electrophoretic mobility shift assay

Fun30-6XHis was serially diluted in 1X HMA buffer (20 mM HEPESpH 7.6, 25 mM KOAc, 5 mM MgAc) containing 0.1% Tween-20. Binding reactions were established in a final volume of 10 µL containing 0.5 X HMA buffer, 0.1 mg/ml BSA, 1 mM DTT, 5 mM AEBSF, and 30 nM Cy3 labeled nucleosome or DNA and 2 µL of Fun30-6xHis diluted to the concentrations described in the figures. Reactions were allowed to equilibrate on ice for 30 min before electrophoresis on 0.5X TBE native polyacrylamide (49:1) gels (5% for nucleosomes, 7.5 % for DNA) at 150 V for 60 min at 4 °C. Gels were scanned on a FLA-5100 laser scanner (Fujifilm) for the Cy3 signal and the nucleosome/DNA bands quantified in AIDA image analysis software (v3.27.001).

2.10. ATPase assays

TAP purified SWI/SNF and Fun30 were incubated at 30°C in 5 µl of reaction buffer (13 mM Na-HEPES [pH 7.9], 3 mM Tris-HCl [pH 8.0], 60 mM KCl, 9 mM NaCl, 7 mM $MgCl_2$, 6% glycerol, 0.6 mM DTT, 0.3 mM EDTA, 2 µM unlabeled ATP, 30 nM [γ-^{32}P]ATP) with 6nM of SWI/SNF and 20 nM of Fun30 in the presence of 2ng of chromatin, ds DNA and ss DNA. Reactions quenched by 2 µl of stop solution (100 mM EDTA, 50 mM Tris-HCl [pH 7.5]). 0.5 µl of the reaction was spotted onto a

polyethyleneimine-cellulose thin-layer chromatography (TLC) plates (JT Baker, Inc.). Inorganic phosphate was separated from un-reacted ATP by running the TLC plates in 0.5 M LiCl and 1 M formic acid. The plate was dried when the solvent front reached three quarters the height of the plate and subjected to autoradiography. For the real time ATPase assay, we used the Invitrogen Pi sensor assay essentially following the manufacturer's protocols as described previously (Ferreira et al., 2007) for a detailed protocol see Appendix section 8.2.

2.11. Glycerol velocity gradient

10 % and 40 % glycerol solutions were prepared containing 40 mm HEPES (pH 7.8), 300 mm NaCl, 0.1% Tween-20, 1mm PMSF, and 1mM DTT. 10 ml of 10 % - 40 % linear glycerol gradients were formed in Beckman SW41 ultracentrifuge tubes using a Gradient Former model from Bio-Rad. The purified TAP-FUN30 complex, catalase, apo-ferretin and BSA were loaded onto the top of the gradients and then centrifuged at 38,000 rpm for 24 h at 4°C in a Beckman SW41Ti rotor. Then 150 µL fractions were collected from the top, and the presence of Fun30 complex was monitored by Western blotting.

2.12. *Sal*I accessibility assay

10 ng of nucleosomes assembled onto the GUB template were incubated with 4 nmol of SWI/SNF or 10 nmol of Fun30 for 1 hour at 30 °C, the binding reactions were then treated with 10 units of *Sal*I for 30 min at 30 °C and processed as described previously (Hassan et al., 2006).

2.13. Nucleosome repositioning/dimer exchange assay

MMTV nucleosome A (NucA) DNA fragments were generated by PCR with the 5' primer labelled with the Cy3 dye to allow substrates to be visualized as described before (Ferreira et al., 2007). Nucleosomes in which H2B was labelled with Cy5 were assembled onto the fragments. Remodelling reactions contained 0.25 pmole of donor nucleosome, 0.75 pmole (3 fold excess) of 146 bp acceptor template (derived from the 601 positioning sequence (Thastrom et al., 2004) assembled with an equimolar ration of $(H3/H4)_2$, 1mM ATP, 3mM $MgCl_2$, 50 mM NaCl, 50 mM Tris-Cl (pH 8.0) and of RSC or Fun30 as indicated. Reactions were incubated at 30°C for 30 minutes and terminated by addition of 0.5 μg of λ *Hind*III digested DNA and quenching on ice. Sucrose was added to a final concentration of 2% as a loading buffer and reactions were run for 3.5 hours on a 0.2× TBE 5% polyacrylamide gel at 4 °C and 300V and visualized using a Fluorimager (FLA5000, Fuji).

2.14. Western transfer of Native gels

Western blotting of Native gels was performed by transfer to a 0.2 μM PVDF membrane (Bio-RAD, USA) in 20 % methanol, 1.44 M Glycine, 0.033 M Tris-base, 0.1 % SDS, overnight at 30 V at 4 °C. Following transfer western blotting was carried out using standard procedures.

2.15. Yeast strains

Wt and knockout *FUN30* viable haploid strains were purchased from EUROSCARF, (Frankfurt, Germany) and were used for the screening experiments. The yeast strains used in this study are summarised in Table 2.1.

Table 2.1. List of stains used in this study

Strain	Strain No.	Genotype	Source
Wt	Y00000	*MAT***a**; ade-; *his3*Δ*1*; *leu 2*Δ*0*; *met 15*Δ*0*; *ura 3*Δ*0*	**Euroscarf, Germany**
Rsc-TAP	BCY211	*MAT***a**, *ade 2*, *arg 4*, *leu 2-3*, *112 trp1-289*,*ura 3-52*,ORF-AP::*URA3*	**Brad Cairns**
Snf6-TAP	YOR290c	*MAT***a**, *ade 2*, *arg 4*, *leu 2-3*, *112 trp1-289*,*ura3-52*,ORF-AP::*URA3*	**M. Carroza**
Snf2Δ	Y01586	*MAT***a**; *his* Δ*1*; *leu 2*Δ0; *et1 5*Δ*0*; *ura 3*Δ*0*; YOR290c::kanMX4 (snf2)	**Euroscarf, Germany**
Fun30-TAP	YAL019w	*MAT***a**; *ade 2*, *arg 4*, *leu 2-3*, *112 trp1-289*, *ura 3-52*, ORF-TAP::*URA3*	**Euroscarf, Germany**
fun30Δ	Y00389	*MAT***a**; *his 3Δ1*; *leu 2Δ0*; *met 15Δ0*; *ura 3Δ0*; YAL019w::kanMX4	**Euroscarf, Germany**
YKL200	-------	*MAT***a**; *ade 2-1*; *ura 3-1*; *his 3-11*,15; trp *1-1*; *leu 2-3*,112; *can 1-100*; UBR::GAL-HA-UBR1 (HIS3)	**Karim Labib lab**
Fun3-degron	-------	*MAT***a**;UBR1::GAL1-HA UBR1::HIS3, *ade 2-1*, *ura 3-1*, *his 3-11*, 15, *trp 1-1*, *leu 2-3*, 112, *can 1-100* Fun30::kanMX	**This thesis**

2.16. Protein extraction

Rupture of yeast cells in the buffer containing TCA preserves protein modifications especially on histones. The method described below is adapted from a protocol described in (Kao and Osley, 2003). 20ml of cells were grown at 30 ℃ with shaking in YPD medium to an OD_{600} of 0.8. then cells are centrifuged at 4500 rpm for 5 min and the cell pellet is immediately resuspended in 5–10 ml of 20% ice cold TCA (trichloroacetic acid). Cells are again centrifuged at 4500 rpm for 5 min and all residual TCA is removed from the cell pellet, which is immediately frozen in an ethanol–dry ice bath and stored at −80 °C. The cell pellet is thawed on ice, resuspended in 0.2 ml of 20 % TCA, and transferred to a 1.5-ml microfuge tube. The cells are broken by bead beating for 2 min at 4 ℃ with acid-washed glass beads (0.2 mm, Sigma). The broken cell pellet is transferred to a new microfuge tube,

avoiding the glass beads, and combined with two 500-µl washes of the beads with 5 % TCA. The lysate will be in ~1.2 ml of TCA at the end of the washes. The lysate is incubated on ice for 10 min or longer, and the precipitated protein is collected by centrifugation at 14,000 rpm for 15 min at 4 °C. The supernatant fraction is aspirated, and the precipitated proteins are briefly recentrifuged and aspirated to remove as much TCA as possible. The precipitated proteins are resuspended in 750 µl of 1X Laemmli sample buffer (0.06 M Tris, pH 6.8, 10 % (v/v) SDS, 5 % (v/v) fresh 2-mercaptoethanol, and 0.0025 % (w/v) bromphenol blue) and 50 µl of unbuffered 2 M Tris is added to neutralize the pH of the lysate. The suspension is boiled for 5 min and clarified by centrifugation at 14,000 rpm for 10 min at room temperature. The clarified lysate is transferred to a fresh microfuge tube and either directly loaded onto an SDS–polyacrylamide gel, or stored at −80 °C.

2.17. Western Blotting

The concentration of protein lysates was measured by Bradford assay using the Bio-Rad protein detection kit (Bio-Rad). Typically, 40µg of the whole cell extract was analyzed in Western blots. Western blot analysis was performed by running a 4-12 % NuPAGE® Novex® Bis-Tris Gels (Invitogen, USA), then transfer of proteins to a PVDF membrane at 200 V for 1 hour in NuPAGE® 1X transfer buffer using a novex transfer chamber. The membrane was then blocked in 50 ml of TBS–Tween (50 mM Tris, 138 mM NaCl, 2.7 mM KCl, 0.05% Tween 20, pH 8.0) containing 5 % milk at 4 °C overnight. The membrane was then incubated with 1:1000 dilution of primary antibodies (listed in Table 2.2) in PBS for 2 hrs at room temperature. The membrane was then washed three times for 10 min each in of TBS–Tween, and incubated for 1 h with 1:3000 of the corresponding secondary antibodies.

Table 2.2. List of primary antibodies

Antibody	Company	Product No
Anti-TAP	Open Biosystems	CAB1001
Anti-Rad53 (yN-19)	Santa Cruz	Sc-6748
Anti-Rad53 (yC-19)	Santa Cruz	Sc-6749
Anti-Rad9 (y-3000)	Santa Cruz	Sc-50442
Anti-Cdc28 (yN-19)	Santa Cruz	sc-6708
Anti-Ubiquitin clone FK2	Biomol	PW8810
Anti-H2B	Upstate	07-371
Anti-H3 K79 di-tri methylation	Abcam	ab3594
Anti-Tubulin	Abcam	ab6161
Anti-H3K4di methylation	Abcam	ab8580
Anti-H3 K4 di-tri methylation	Upstate	ab6000
Anti-H2AZ	Cell Signalling	2718

2.18. Screening for drug sensitivity and sensitivity to irradiation

1:10 dilution of log phase (OD_{600} ~ 1.5) cultures were inoculated into normal YPD and YPD plates containing the different chemicals as follows; 1M NaCl, 150 µg/ml 6-Aza uracil (Sigma, Germany), 20mM caffeine (CALBIOCHEM, Germany), 5 mM Hydroxyurea (HU) (Sigma, Germany), 0.0050 % Methyl Methanesulfonate (MMS) (Sigma, Germany), 0.25 % 5-Fluoroorotic Acid 5-FOA, (Sigma, Germany), 15 mM 3-Amino-1,2,4-triazole (3-AT) (Sigma, Germany), and with 20 % lactose or galactose/raffinose for the YPlac and YPRG. For UV and IR resistance experiment plates were exposed to 100 J/m2 of UV from UV cross linker for different time intervals and 100 Gy from Varian Clinac® 2100C linear accelerator. For termination assay yeast plates were prepared containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2 % (w/v) gal, and 20 mg/ml ethidium bromide (Johnston and Davis, 1984).

2.19. Flow cytometry for cell cycle analysis

Flow cytometry was performed on cultures that had been synchronised following arrest with alpha factor to monitor their DNA content. The most commonly used dye for DNA content/cell cycle analysis is propedium iodide (PI). It can be used to stain whole cells or isolated nuclei. PI intercalates into the major groove of double-stranded DNA and produces a highly fluorescent adduct. In brief; 25ml cultures of wild type and *fun30*Δ cells were grown overnight. The cultures were then diluted to $OD_{600}\approx$ 0.1 and incubated at 25 °C with shaking. α-Factor was then added gradually to a final concentration of 0.75 µg/ml to arrest growth, and the cultures were further incubated for 2.5 hrs. α-Factor was then removed by filtration followed by 3 washes of d.H_2O. The washed pellets were resuspended in fresh 25 ml YPD and immediately 1ml of the cultures was taken as zero point and fixed with 70% ethanol.

The remaining of the cultures were incubated at the temperatures indicated and 1ml of culture removed and fixed at specific time intervals (typically 20 min each), also about 20 µl of each time point was collected for calculation of the percentage of budded cells under light microscope. Fixed cells were pelleted and resupended in 1ml of 50mM Tris-HCl pH 7.8 and 20µl of 10mg/ml RNase. The resupended pellets were incubated for at least 6 hours at 37°C with shaking. After that the RNase treated cells were pelleted and washed once with washing buffer (200mM Tris-HCl pH 7.5, 211mM NaCl, 78mM MgCl2). Then the pellets were resuspended in 0.5ml of the above mentioned buffer and 50µl of 1mg/ml PI was added. Samples were sonicated for 5-7 sec to separate the cells. Before loading into the FACS machine cells were further diluted in 50mM Tris-HCl pH 7.8 if the count of the cells exceeded the threshold (20000 cells), . Then the samples were analyzed using FACS Calibur (BD,

USA) and the filter FL2 was used to detect PI. Data were then processed using CellQuest software.

2.20. Degron construction

The ability to conditionally inactivate proteins is a powerful method for investigating their function. One simple way to achieve this is by fusing a protein sequence that targets a protein for proteasome mediated degradation (Sanchez-Diaz et al., 2004).
In this case the N terminus of the encoded protein sequence was fused to heat inducible degron cassette. Degradation requires recognition of the degron cassette by the evolutionarily conserved Ubr1 protein, which is associated with a ubiquitin-conjugating enzyme which will be activated when cells are shifted from 24 °C to 37 °C (Sanchez-Diaz et al., 2004) see Fig. 2.2. One quick approach is to create the degron fusion in a haploid strain such as YKL200, in which the only copy of the UBR1 gene is under the control of the GAL1, 10 promoter. For a detailed protocol see Sanchez-Diaz et al. (2004).

To create *FUN30*-degron we used a one-step PCR (See Fig. 2.3) method descried in Sanchez-Diaz et al. (2004). The degron cassette is inserted into the chromosomal locus of *FUN30* by integration of a DNA cassette that is amplified by PCR and subsequently used to transform yeast cells (Fig. 2.3). To direct integration to the correct site, the oligonucleotides chosen for the PCR reaction ensure that the ends of the cassette are identical to the chromosomal locus of the target gene.

Forward primer:

5' GTAAGGAACG TAAACAAGAA AAAGAGAGAA AATACGCTAT AGTTGAAAAC ATTAAGGCGCGCCAGATCTG 3'

Reverse primer:

5' TCGGGCACTT GCACTACGTC ATCCTCATCA TTTGAATGCG AACCACTCATGGCACCCGCTCCAGCGCCTG 3'

The cassette contains three components: the *kanMX* gene, followed by the *CUP1* promoter, and then the degron itself. The kanMX gene acts as a selectable marker and confers resistance to the antibiotic drug G418. The *CUP1* promoter is used to express the degron, because the integration will disrupt the endogenous promoter, and induction of *CUP1* can be induced by the addition of $CuSO_4$ to the medium. Two 100-µl PCR reactions we set up to prepare DNA for each yeast transformation using the following conditions:

One cycle: 94°C, 2 min

30 cycles: 94°C, 1 min; 55°C, 1 min; 72°C, 5 min

One cycle: 72°C, 10 min; hold at 4°C until the samples are removed.

DNA was pooled together from the PCR reactions and purified using the QIAquick PCR purification kit according to the manufacturer's instructions. The DNA was eluted with 30 µl of 1x TE, pH 8.0 and store at -20°C. YKL200 were transformed with the PCR product, and selection was performed using YPDCuG200 Plates. This is followed by screening for clones that have the right integration, using four different 20-mer primers to check whether the degron cassette has integrated into the correct chromosomal location. Two of these primers are specific for a *FUN30* gene: One is chosen from the Watson strand about 500 bases upstream of the initiator ATG (this is primer A); the other is chosen from the Crick strand about 500 bases

downstream of the ATG (this is primer B). The remaining two primers correspond to sequences in the degron cassette. The following sequences were used:

Primer A, 5'-TCCTACCAGATTCCCGTCAT-3'

Primer B, 5'-AATGGCACCGGTTTGTCTTT-3'

Primer C, 5'-CTGGTGCAGGCGCTGGAGCG-3'

Primer D, 5'-CGCTCCAGCGCCTGCACCAG-3'

The next step after identifying a degron strain with the correct integration was to investigate viability at 37°C in the presence of high levels of the Ubr1 protein. Strains were tested for growth with and without induction of Ubr1 at 24°C or 37°C. Because 24°C is intended to be a "permissive" condition, cells were examined on YPDCu and YPGCu at this temperature. Because 37°C should be "restrictive," but only in the presence of high levels of Ubr1, cell growth was tested at this temperature on YPDCu and YPG plates. YPG plates were used because they lack CuSO4 and since we were aiming to deplete the protein, thus it would help to reduce transcription from the CUP1 promoter. Furthermore, the functionality of the degron was also confirmed by Western blot analysis (Fig. 2.3). After determining the correct clones, glycerol stocks were prepared. To deplete *FUN30* cells were incubated at 37°C for 30 min before analysed in FACS analysis and Western blotting as mentioned previously.

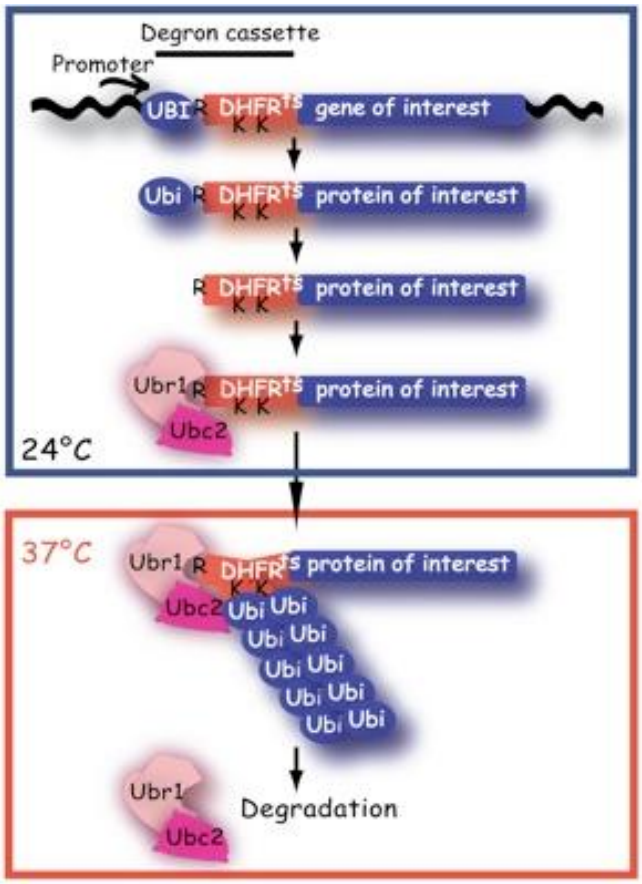

Figure 2.2. Representation of the regulated destruction of protein by fusion to the heat inducible degron. Genetic analysis of protein function is greatly aided by the ability to rapidly inactivate the target protein. In the heat-inducible degron system, the protein sequence can be fused to other proteins, in principle allowing the stability of the fusion protein to be controlled by a simple shift in temperature. The efficient degradation of such fusions can be achieved by increasing expression of Ubr1, the E3 ligase that targets the degron for ubiquitylation at 37°C. Modified from http://web.mac.com/karimontheroad/CellCycleGroup/Degron.html

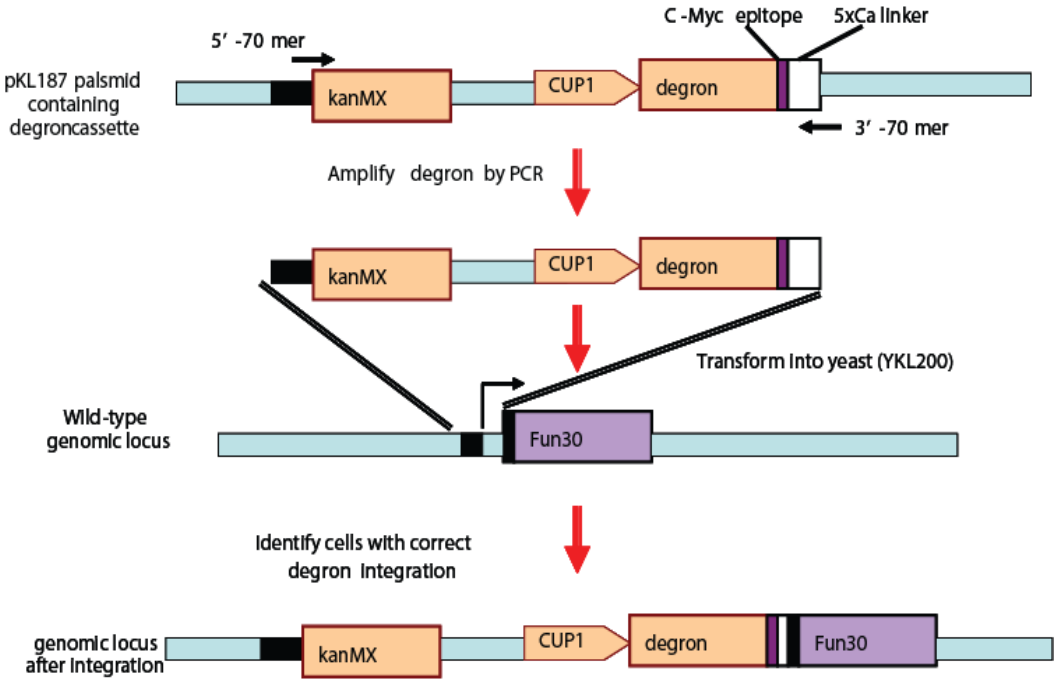

Figure 2.3. Construction of the Fun30-degron strain by one-step PCR method. The plasmid pKL187 a gift from Karim Labib was the template for the PCR reaction. The cassette was amplified with *Kan*MX selectable marker, which had also the CUP1 promoter expressing the degron cassette, followed by a single copy of the c-myc epitope and a short linker sequence (5 repeats of the amino acid pair Gly-Ala. The linker allowed the target protein and the degron to fold independently of each other.

2.21. Chromatin Immunoprecipitation (ChIP)

Preparation of IP DNA: 50ml of TAP-Fun30 yeast culture was grown to OD_{600} 0.25. 37% formaldehyde was then added to a final concentration of 1%, incubated at room temperature for 15min with gentle shaking. Then the crosslinking reaction was quenched by adding 2M glycine to a final concentration of 125mM, mixed by shaking and incubated at room temperature for 5min. Then cells were pelleted by centrifugation at 3000 rpm for 10min. This is followed by washing the pellet with 20ml cold TBS three times. Cells were resuspended in the TBS that remains in the bottle after decanting (about 0.5mL), and transferred to a 2.0ml tube. Cells were then centrifuged in a cold microcentrifuge for 10-30sec. After that, cells were resuspended in 400 µl ChIP lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1% TritonX-100, 0.1% Sodium Deoxycholate, 1mM EDTA + protease inhibitors). An equal volume (about 0.5ml) of acid washed glass beads (425-600 micron, Sigma) was added and the cell walls broken down by bead beating for 3 x 40sec using Mini-Bead-Beater (Biospec), with 40 sec pauses between runs, in the cold room.

The tube caps were then removed and the tube bottoms pierced with a hot 20 G 1.5 needle. Pierced tubes were put into another 1.5ml tube and spun in a cold centrifuge at top speed for 30sec. The insoluble pellets were emulsified with their supernatants and transferred to new 1.5ml round bottom tubes. Extracts were sonicated in water bath sonicator at level 5, 100% duty, 4 X 30 sec with 30 sec pauses on ice between runs (sonicator output should be approximately 20-25 when sonicating samples). Extracts were then clarified by spinning in a refrigerated microcentrifuge for 10 min at top speed. Supernatants were transferred to 1.5 ml tubes and centrifuged again for 5-10 min at top speed to remove any debris. Fragmentation was then checked on an agarose gel.

Pull-down of extracted DNA: 30µl bed volume of IgG-Dynabeads (Dynal, USA) were added to 200µl of extracted sheared DNA and incubated on a rotating wheel for 2hr at 4°C. At the same time controls (i.e. no Ab control; untagged strain + Ab) were also prepared. Using a magnetic stand the beads were separated from the supernatant and washed two times with 1ml of ChIP lysis buffer, followed by another two-times with 1ml lysis buffer containing 500mM NaCl, and two-times with ChIP washing buffer (10mM Tris-HCl pH 8.0, 250mM LiCl, 0.5% NP-40, 0.5% Sodium Deoxycholate, 1mM EDTA). This was followed by a final wash with 1ml TE buffer. Beads were then resuspended in 50µl of ChIP elution buffer (50mM Tris pH 8.0, 1%SDS, 10mM EDTA) and incubated at 65 ° C for 30 min. Then the eluted DNA/protein was then subject to reverse cosslinking by adding 120µl of TE buffer containing 1% SDS, and incubated for overnight at 65 ° C. Then 7.5 µl of 20 mg/ml of protinase K and 2µl of 10mg/ml of RNase were added and incubated at 37° C for 2hr. DNA was extracted and cleaned using Qiagen PCR purification kit according to manufacturer's instructions. Finally, DNA was eluted in 40µl of EB buffer.

Sequencing of the IP DNA*:* The ends of the sheared DNA were converted to blunt ends by adding 1ul (about 0.25 units) of T4DNA polymerase per 1µg of sheared IP DNA, the mixture was incubated for 15min at 12°C, and EDTA was added to final concentration of 10mM. Then DNA was cleaned using Qiagen PCR purification kit and resupended in 10µl of d.H_2O. The blunt ended DNA was ligated into peGFP after digestion with *Sma* I/*SAP* and transferred into DH5α. Then colonies containing insertions were identified by restriction enzyme digestion. Recombinant plasmids containing the blunt IP DNA were extracted by Qiagen mini prep kit. And the vector was sent for sequencing at the sequencing facility at University of Dundee.

Preparation of samples for hybridisation to Tiling arrays*:* The purified IP-DNA was amplified using Sigma GenomePlex® Complete Whole Genome Amplification (WGA) Kit according to manufacturer's instructions. This kit provided a method to generate a representative ~ 500-fold amplification of IP-DNA. The amplified DNA was quantified using Pico-drop spectrometer and conventional 1.5% agarose gel electrophoresis. Samples were amplified so that the final concentration was about 250ng/sample and 10µg in total. Amplified DNA samples were sent to Nimblgene and processed on a standard tilling array with 380,000 elements. Data was normalized and saved in MYSQL database.

Data analysis: Data analysis and plotting were generated using scripts written in Python software. For ARS and promoter analysis data were generated 1500 bp on either side, whereas in the transcription factors analysis were obtained by using 50bp enrichment on either side of the transcription factor binding sites. After background subtraction, the ratio of immunoprecipitation-enriched signal to whole-cell extract signal at each spot on the microarray was calculated, the spots were sorted by ratio, and a percentile was assigned to each spot (e.g., the 90th percentile denotes that 90% of all spots on that microarray have lower ratios). The values for each spot were used to generate a list of intergenic sequences ordered by degree of enrichment.

Alignment to ARS elements or promoters: Alignment methods aim to identify functional elements by a multiple local alignment of all sequences of interest use an optimization procedure based in probabilistic sequence models to find statistically significant motifs. These approaches use a combination of enumerative and

alignment methods such as hierarchical clustering so as to have significant improvement in the identification of regulatory elements.

The samples were competitively hybridized to a microarray containing all of the intergenic sequences in the yeast genome. The values for each spot were used to generate a list of intergenic sequences ordered by degree of enrichment. The approach to the data analysis in this thesis was shaped by the need to determine the level of Fun30 occupancy and the importance of considering broad relationships among genes targeted by it. To this end, we used Python program that searches for enrichments among Fun30 targets of genes in functional categories defined by a single attribute, such as a biochemical activity or subcellular localization. The *Saccharomyces* Genome Database (SGD) maintains the most current annotations of the yeast genome (*see* http://www.yeast-genome.org/). The SGD FTP site contains the DNA sequences annotated as intergenic regions in FASTA format (available at ftp://genome-ftp.stanford.edu/pub/yeast/sequence/genomic_sequence/intergenic/), indicating the 5′ and 3′ flanking features. These files can be used to extract upstream intergenic regions.

Python program combines the occupancy data with information in the SGD database (Costanzo et al., 2001) to identify the enrichments and calculate a statistic describing the significance of each enrichment. There are two inputs to the program: the ranked list of ORFs from the genome-wide localization and a list of functional categories and the genes in each category as annotated in SGD. A cut-off corresponding to the maximum statistical enrichment for that category was determined.

Ch.III: The Snf2 homolog Fun30 acts as a homodimeric ATP-dependent chromatin-remodelling enzyme*

3.1. Introduction

Historically Fun30 was identified as a result of sequencing of six potential open-reading frames (ORFs), provisionally named YAL001-006 within a 42 kb segment of chromosome I (Kaback et al., 1984). Four of these ORFs can be aligned with formerly recognized FUN (Function Unknown Now) transcripts: Fun28 with YAL006, Fun29 with YAL004, and Fun30 with YAL001 and Fun31 with YAL002 (Kaback et al., 1984). Disruptions of these genes indicated that all were not necessary for growth on rich medium at 30°C (Clark et al., 1992; Barton and Kaback, 1994). Further characterisation of *fun30* deletion mutants revealed temperature sensitivity and resistance to UV irradiation (Barton and Kaback, 1994).

More recently, it has been shown the deletion of *FUN30* leads to sensitivity to the topoisomerase I poison camptothecin and to severe cell cycle progression defects when the *ORC5* is also mutated (Neves-Costa et al., 2009). Moreover, it has been reported that *FUN30* plays an important role in promoting silencing in the heterochromatin-like mating type locus HMR, telomeres and the rDNA repeats (Neves-Costa et al., 2009). Using Chromatin immunoprecipitation assay the same study demonstrated that Fun30 binds both at the boundary element and within the silent HMR locus. Interestingly, mapping of nucleosomes *in vivo* using micrococcal nuclease showed that deletion of *FUN30* leads to changes of the chromatin structure at the boundary element (Neves-Costa et al., 2009).

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Furthermore, the ATPase activity was reported to be essential for these roles of Fun30 (Neves-Costa et al., 2009). As a result of these observations it has been proposed that *FUN30* is directly involved in silencing by regulating chromatin structure within or around silent loci (Neves-Costa et al., 2009). The human homolog of Fun30 protein (SMARCAD1) is annotated as containing CUE motifs (Neves-Costa et al., 2009) that may be involved in recognizing ubiquitin (N-terminal to the Snf2 related region) see Fig. 1.3. Here in this chapter we will address the purification and characterization of Fun30 as a chromatin remodelling enzyme.

3.2. Results

3.2.1. Purification and characterization of the Fun30 protein complex

To better understand the function of the Fun30 protein and study its biochemical activity, we set out to purify the Fun30 protein complex from *Saccharomyces cerevisiae* using Tandem Affinity Purification (TAP) technique (Puig et al., 2001).

A strain in which the endogenous *FUN30* gene had been tagged was purchased from EUROSCAR (EUROSCARF, Germany). Fun30 purified from extracts of this strain as described in section (2.1) was visualised by silver staining following gel electrophoresis. A major band at 128 KDa corresponding to the size of Fun30 (Fig. 3.1A) can be seen, suggesting that Fun30 is not a component of a large stable complex. When the TAP purified protein was subjected to gel filtration chromatography it was found to elute in fractions consistent with a molecular weight of approximately 250 KDa (Fig. 3.1B). Purified Fun30 was also fractionated on a 10%-40% glycerol gradient by velocity centrifugation (Fig. 3.1C). Again the size of the TAP-purified Fun30 protein peak was estimated to be 250 KDa. Recombinant Fun30 expressed in *E. coli* was found to exhibit similar behaviour following glycerol gradients and gel filtration. This indicates that no other yeast proteins are required for this anomalous behaviour (data not shown).

To investigate this further, the TAP-tagged strain was transformed with a GAL inducible HA tagged Fun30 expression plasmid. This resulted in a strain expressing Fun30 tagged with both a hemagglutinin A (HA) epitope and the TAP tag. Following induction, total cell protein was isolated from both the singly and doubly tagged strains and the protein concentration measured by Bradford assay. Immunoprecipitation with the TAP antibody enriched for TAP-Fun30 on the beads in

both strains (Fig. 3.2A). Probing the immunoprecipitates with an antibody against the HA-epitope after an IP with the TAP antibody showed that HA-Fun30 was precipitated with the TAP antibody only in the co-expressing strain (Fig. 3.2A, second panel lane 3). Moreover, the reciprocal immunoprecipitation confirms these findings; following immunoprecipitation with the HA antibody, TAP-fun30 is only detected in the co-expressing strain. These observations indicate that the two differently tagged forms of Fun30 interact in yeast nuclear extracts. As it is possible that additional proteins might mediate this interaction within an extract, we next investigated whether highly purified Fun30 prepared with different tags and from different sources could interact.

To this end, we expressed a recombinant form of Fun30 in *E. coli* that contained a C-terminal histidine tag (Fun30-6xHis). Fig. 3.2B shows that when purified TAP-Fun30 and Fun30-6xHis were mixed, and Fun30-6xHis pulled down on Ni^{2+} beads both forms of the protein were precipitated. This indicates that Fun30 is capable of directly interacting with itself. All together, with the observation that the size of the native TAP-Fun30 complex is twice that of a Fun30 monomer, these data provide strong evidence that the Fun30 protein exists predominantly as a homodimer of approximately 250 KDa.

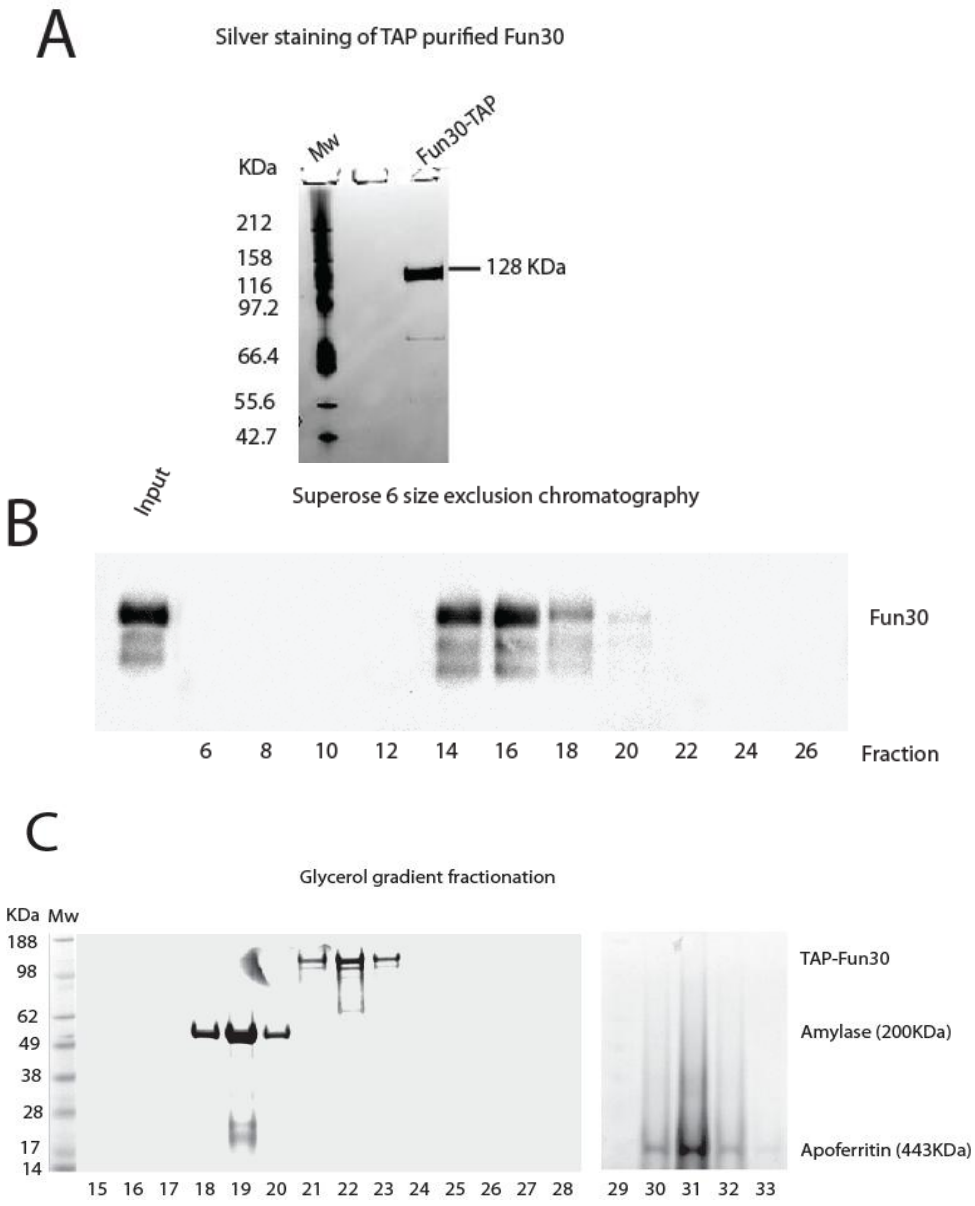

Figure 3.1. Purification of the Fun30 complex. (A) SDS-PAGE and silver staining of TAP-purified Fun30. (B) Superose-6 chromatography of TAP-purified Fun30. Fractions containing Fun30 were detected by Western blotting with anti-TAP antibody. The Fun30 peak (fraction 16) corresponds to a molecular weight of 250 kDa calculated from a standard curve of molecular weight standards run on the same column. (C) Fun30 was resolved by 10%-40% glycerol gradient sedimentation together with amylase and apoferritin as molecular weight markers. Coommassie Blue staining following SDS page of the fractions indicated where each protein elutes. The elution profile for Fun30 peaked in fraction 22 consistent with a complex of about 250 kDa.

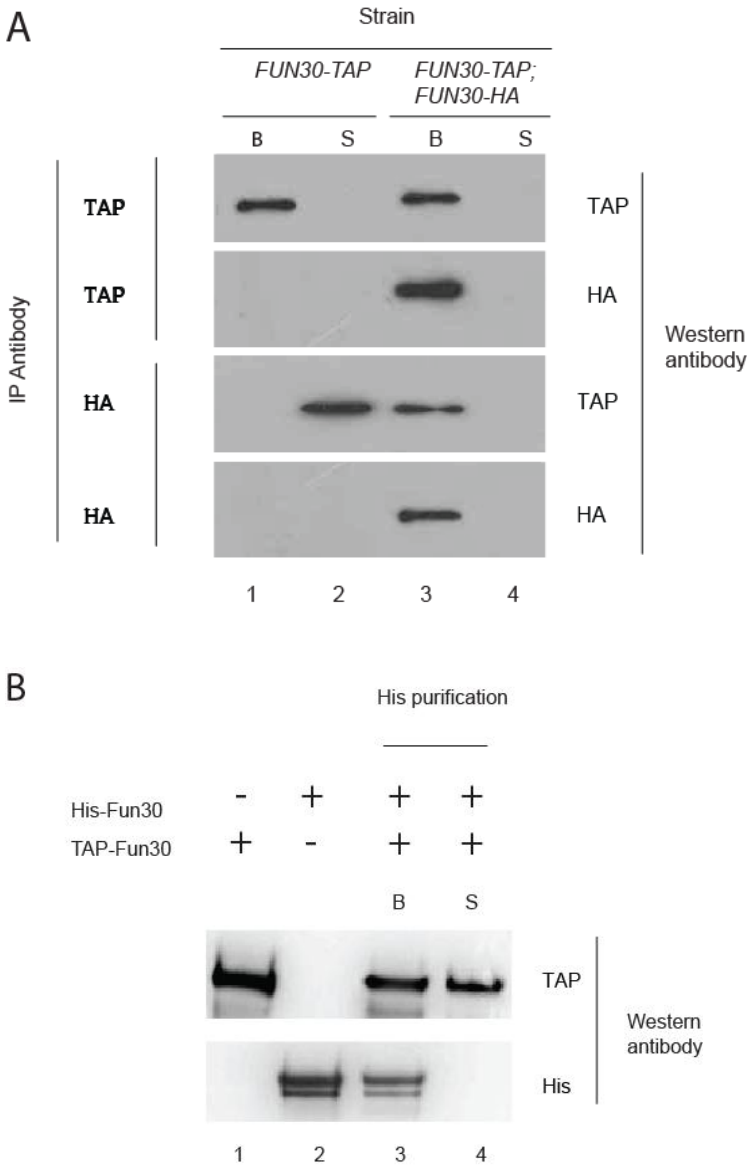

Figure 3.2. TAP-purified Fun30 is a homodimer. (A) Western blots of Fun30 recovered following reciprocal co-immunoprecipitation from a yeast strain expressing either TAP tagged Fun30 (Lanes 1 and 2) or both HA and TAP tagged Fun30 (Lanes 3 and 4). Detection of HA tagged protein in the bound fraction (B) following TAP IP and TAP following HA IP (Lane 3) indicates that the two forms of Fun30 interact. Immunopurification effectively removed Fun30 from the supernatant (S). (B) Preparations of Fun30 purified by TAP-Tag and His-Tag were mixed together and subject to IP with Ni agarose beads. The presence of TAP tagged Fun30 on the Ni beads indicates that these two proteins can interact.

3.2.2. Fun30 is an ATP-dependant chromatin remodelling protein

All Snf2 family proteins studied to date have ATPase activity that is stimulated by the presence of DNA and/or chromatin. The presence of the related helicase domains in the Fun30 protein, raise the possibility that this protein also has the ability to hydrolyze ATP and remodel chromatin structure. To investigate whether Fun30 also has ATPase activity, TAP-Fun30 was incubated with radio-labelled ATP in the presence or absence of DNA or chromatin. Radio-labelled Pi was then separated from ATP by thin layer chromatography (TLC). This showed that the ATPase activity of Fun30, like SWI/SNF is stimulated by both DNA and chromatin (Fig. 3.3A). Using a real time assay for phosphate release, the specific activity of TAP-Fun30 was also found to be comparable to that of the RSC complex (Fig. 3.3B). This indicates that the Fun30 complex we have purified from yeast has an ATPase activity comparable to that of other chromatin remodelling enzymes. This suggests that it could potentially remodel the structure of chromatin in the presence of ATP.

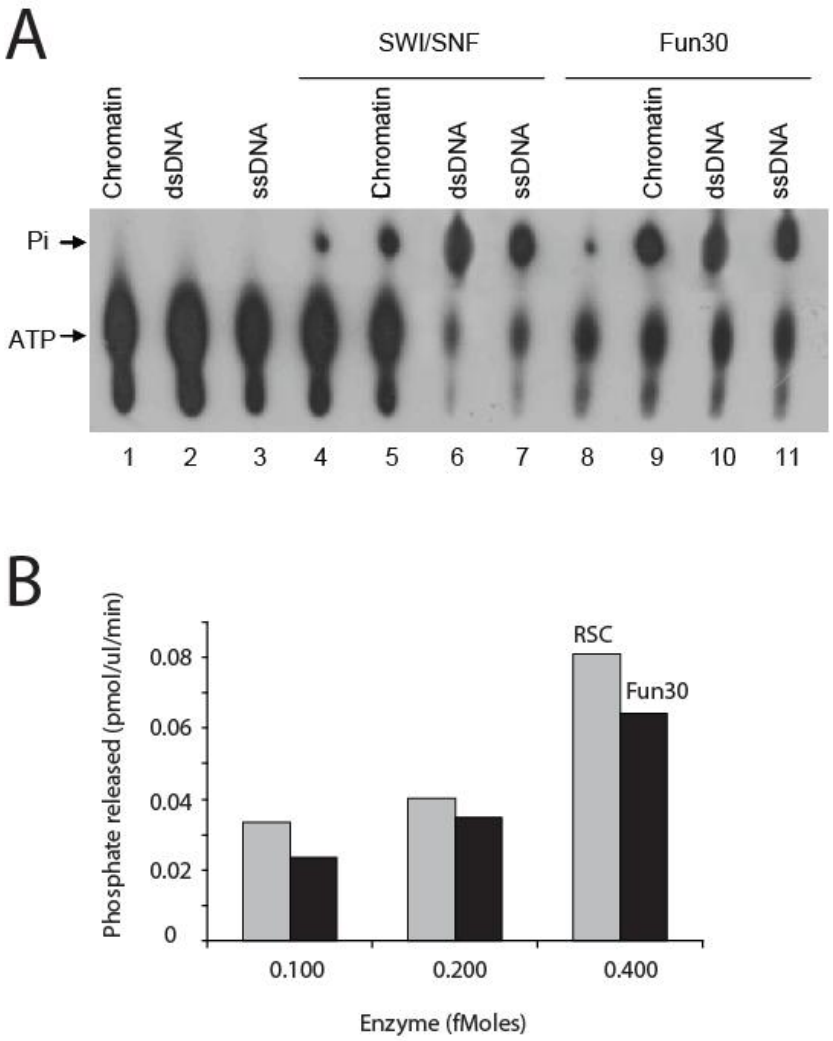

Figure 3.3. Fun30 has ATPase activity. (A) Thin-layer chromatography (TLC) analysis showing ATPase activity of the Fun30 complex. The ATPase activity of Fun30 ~12nM (lanes 8-11) is compared to that of the SWI/SNF complex ~ 5nM (lanes 4-7) using 2ng of either single-stranded (ss) or double-stranded (ds) DNA, or 2ng of HeLa chromatin as the substrate. In lanes 1-3 no chromatin remodelling proteins are added, and in lanes 4 and 8 no substrate is included. (B) Fun30 has a specific activity comparable to RSC. Rates of ATP hydrolysis were assessed using a real time ATPase assay using the indicated quantities of enzyme in the presence of 2ng chromatin.

3.2.3. Fun30 binds to DNA, mononucleosomes, and nucleosome arrays

In order to remodel chromatin, chromatin-remodelling complexes have to be able to recognize and bind to their substrate as a first step. Many chromatin-remodelling complexes have also been shown to interact with DNA and histones (Hassan et al., 2001). For example, the yeast SWI/SNF complex has been shown to bind to naked DNA in an ATP-independent manner, with an affinity in the nanomolar range. The affinity of SWI/SNF for nucleosomes is slightly higher than that for naked DNA, which can be attributed to additional interactions of SWI/SNF with the core histones. Cellular chromatin consists of arrays of nucleosomes rather than mononucleosomes.

Therefore, we investigated the ability of Fun30 to bind chromatin using a DNA fragment of approximately 2.5 kb in length that was immobilized to paramagnetic beads either as free DNA or following chromatin assembly. We assembled nucleosomes onto the G5E4 DNA template using salt dilution. This DNA template which contains 5 Gal4-binding sites upstream of the adenovirus 2 E4 minimal promoter, flanked on both sides by five 5S rDNA nucleosome positioning sequences, was end-biotinylated, reconstituted, and immobilized onto streptavidin paramagnetic beads (Dynabeads). After various incubations, the washed immobilized nucleosome arrays were assayed by Western blots for the presence of the Fun30 and/or SWI/SNF complex protein using the anti-TAP antibody. Fun30 was found to be capable of binding to DNA and chromatin fragments with comparable efficiency (Fig. 3.4A, lanes 7-10).

To gain further insight into the nature of the complex between Fun30 and nucleosomes, quantitative EMSAs were performed using Fun30-6xHis and mononucleosomes assembled at defined locations on DNA fragments derived from

the 601 sequence (Lowary and Widom, 1998). Titration of Fun30-6xHis into reactions containing Cy3-labelled mononucleosomes possessing 47 bp of linker DNA on one side (0W47) resulted in a shifting of the nucleosome to a slower migrating species in a fashion dependent on the concentration of Fun30 (Fig. 3.4B). This shift is diffuse and appears to go through a transition from faster migrating (Fig. 3.4B, lane 4) to slower migrating complexes (Fig. 3.4B, lane 6). This suggests that more than one molecule of Fun30 can bind to a single nucleosome with high affinity and possibly in a cooperative fashion, and this is again consistent with Fun30 being able to associate with itself.

Previous work has shown that some chromatin remodelling enzymes interact with linker DNA flanking the nucleosome core (Whitehouse et al., 2003; Zofall et al., 2004; Strohner et al., 2005; Stockdale et al., 2006). We sought to test whether this was also the case for Fun30 by measuring the binding affinity of Fun30 for nucleosomes that had no flanking linker DNA (0W0). Although we observed that Fun30 could also bind to nucleosomes lacking linker DNA, higher concentrations of Fun30 were required to observe the same degree of binding (Fig. 3.4C). This suggests that the association of Fun30 with chromatin is influenced by interactions with DNA flanking the nucleosome core. All these data together demonstrate that the Fun30 protein can stably bind to DNA, mononucleosomes, and nucleosome arrays

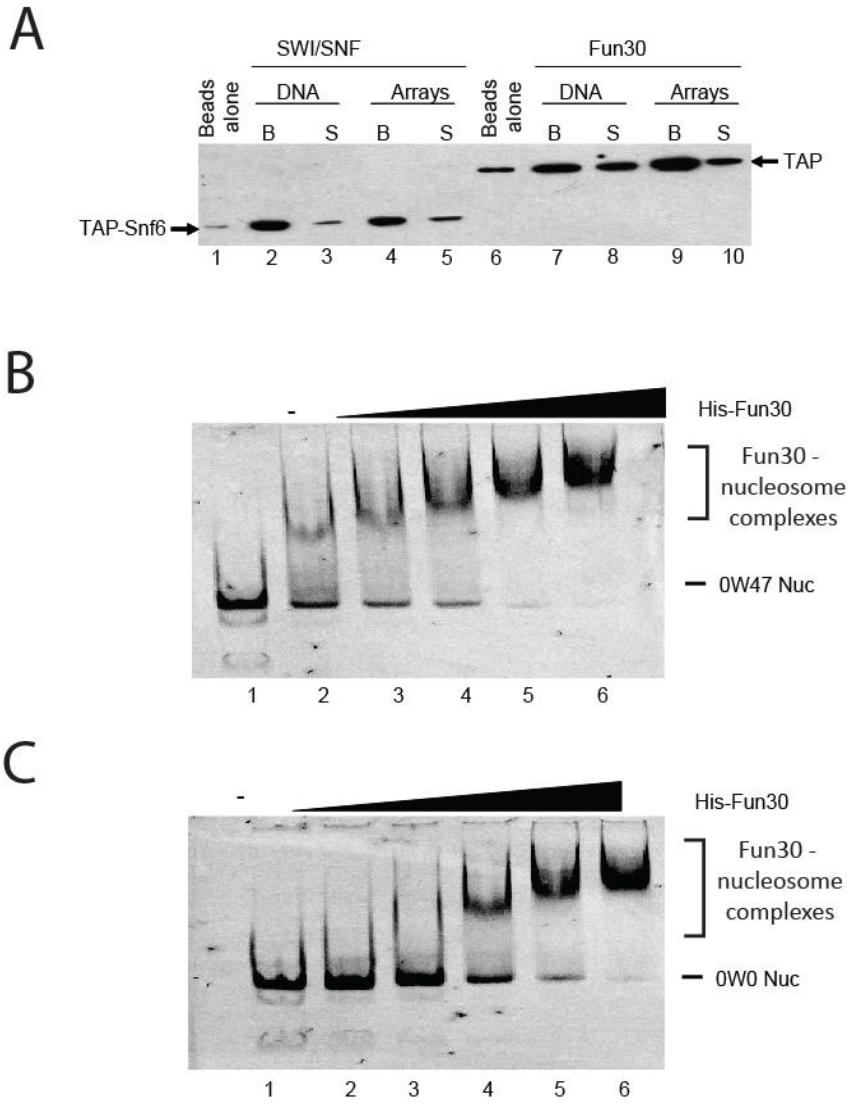

Figure 3.4. The Fun30 binds DNA and chromatin. (A) Immobilized G5E4 (either DNA or reconstituted into nucleosomal arrays), generated as described in the material and methods, was incubated with equal amount of Fun30 (lanes 7-10) or the SWI/SNF complex (lanes 2-5, as control) based on anti-TAP Western blotting normalization. The amount of bound protein (SWI/SNF complex or Fun30) was determined by separating the supernatants (S) from the beads (B), washing the beads, and running them on a 12% SDS gel followed by Western blot analysis using the anti-TAP antibody for detection of the proteins. The background binding of SWI/SNF or Fun30 to the magnetic Dynabeads alone are shown in lanes 1 and 6, respectively. (B) Nucleosomes were assembled on the fragment 0W47 in which the 601 nucleosome positioning sequence directs assembly of a nucleosome such that it is flanked by 47 bp of linker DNA on one side. Incubation of 30 nM of 0W47 nucleosomes with increasing concentrations of 6 his Fun30 (28 nM – 1µM, lanes 2-6) resulted in gel shifted species (Nuc/Fun30). (C) Fun30 association with chromatin is influenced by interactions with DNA flanking the nucleosome core. Nucleosome cores assembled onto the DNA fragment 0W0 were incubated with Fun30 as described for Figure 4B. The binding as assessed by the disapperance of unbound nucleosomes is reduced in comparison to either free DNA or Nucleosomes bearing linker DNA.

3.2.4. Fun30 has ATP-dependent chromatin remodelling activity

To investigate whether the ATP hydrolysis by the Fun30 protein observed earlier could lead to chromatin remodelling by this complex, we first utilized a restriction enzyme accessibility assay. In this assay, a 183-bp long "GUB" DNA template (Hassan et al., 2006) was reconstituted into mononucleosomes. We analyzed the ability of the restriction enzyme *Sal*I to cleave its site in the middle of the "GUB" nucleosomal DNA in the presence or absence of Fun30 and ATP (Fig. 3.5A). An ATP-dependant increase in the restriction enzyme digestion of the template by this enzyme is indicative of nucleosome disruption by the remodelling protein. However, when nucleosomes assembled onto this fragment were incubated with either SWI/SNF or Fun30, *Sal*I was able to gain access to and cleave a significant proportion of the templates (Fig. 3.5B). This effect required the presence of ATP as no increase in cleavage was detected when ATP was omitted (Fig. 3.5B, lanes 7 and 10). These observations indicate that like other Snf2 family proteins Fun30 can act to increase access to DNA within nucleosomes.

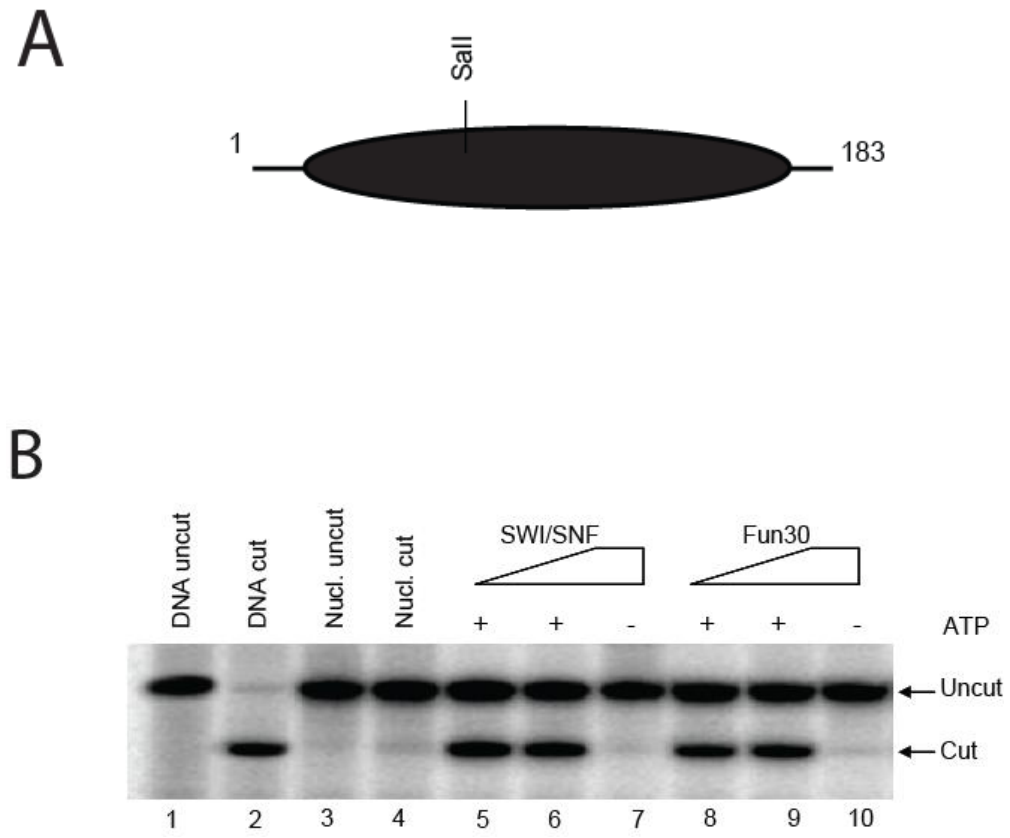

Figure 3.5. Fun30 is an ATP-dependent chromatin remodelling enzyme. (A) Schematic illustration of the template used for restriction enzyme accessibility assays. (B) Restriction enzyme accessibility assay. Increasing amounts of SWI/SNF (~ 2-8nM, lanes 5-7) and Fun30 (~ 10-40nM, lanes 8-10) based on normalization were added to approximately 10 ng of the GUB template in the presence or absence 2mM of ATP as indicated. The binding reactions were then treated with 10 units of *Sal*I for 30 min at 30 °C and the proportion of DNA cleaved assessed by electrophoresis.

3.2.5. Fun30 can slide nucleosomes *in cis* and exchange H2A-H2B dimer in an ATP dependent fashion

Although the restriction enzyme accessibility assay described above provides evidence of an alteration to chromatin structure, it does not provide significant insight as to how the structure of chromatin is altered. To investigate this further, we initially tested the ability of Fun30 to catalyze the repositioning of nucleosomes from one location to another. Although we could detect some repositioning activity this was relatively modest (data not shown). We next sought to study the ability of Fun30 to direct the removal of histone H2A/H2B dimers from nucleosomes and their transfer onto tetramers of histones H3 and H4 assembled on DNA. To do this, we made use of an assay in which histone dimers are labelled through attachment of the fluorescent dye Cy5 (Bruno et al., 2003). When chromatin was subject to remodelling with increasing concentrations of Fun30, Cy5 signal was removed from the nucleosomes and accumulated on the acceptor (Fig. 3.6, lanes 3-6). When the donor nucleosomes were assembled such that they were asymmetrically positioned with 54 bp on one side and 18bp on the other some repositioning of nucleosomes on the donor DNA could also be observed (Fig. 3.6A, lane 6).

However, the extent of repositioning was less than that observed with the RSC complex (Fig. 3.6A, lanes 8-11). This suggested that the Fun30 complex was more efficient in exchanging histone dimers than repositioning nucleosomes. This effect was most striking when nucleosomes were assembled onto donor fragments such that they had 54 bp extensions on either side (Fig. 3.6C). In this case, RSC caused extensive repositioning of these nucleosomes, but Fun30 had relatively little effect, yet in both cases dimer exchange was comparable. When we used donor

nucleosomes that had 54 bp extensions only on one side, again dimer exchange was observed while nucleosome repositioning was limited (Fig. 3.6B).

These results together show that the Fun30 complex can remodel chromatin by moving nucleosomes *in cis* and that it is particularly effective in driving the exchange of histone dimers from one DNA fragment to another.

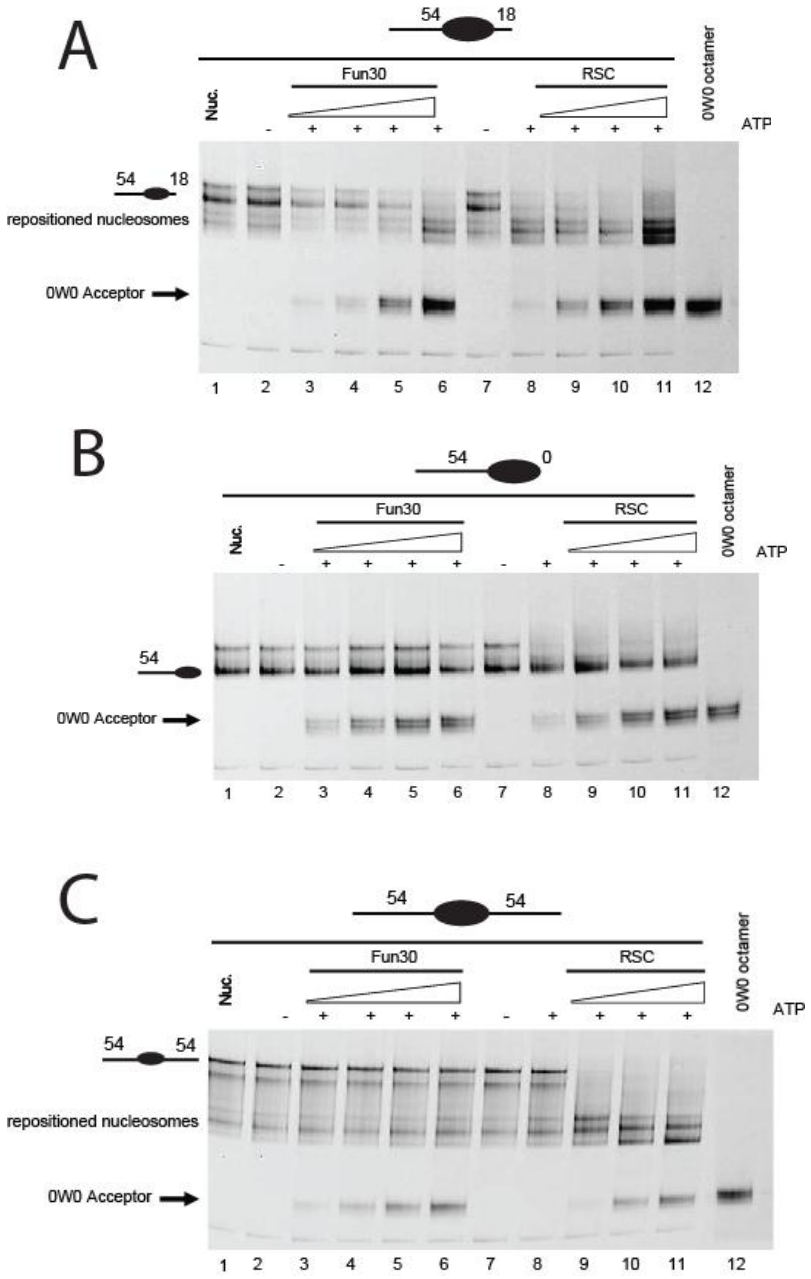

Figure 3.6. Fun30 has higher activity in histone dimer exchange than nucleosome repositioning. 10µl reactions containing 0.25 pmoles of nucleosomes (250 nM) in which H2B is fluorescently labelled with Cy5 assembled at the MMTV nucleosome A (NucA) positioning sequence flanked by the indicated lengths of linker DNA were incubated with Fun30 (100, 200, 300, 400 pM, lanes 3-6) or RSC (100, 200, 300, 400 pM, lanes 8-11) in the presence of 0.75 pmoles histone tetramers assembled onto 147bp DNA (0W0). In each panel, Lane 1 contains nucleosomes assembled on the appropriate donor DNA fragment and lane 12 contains the 0W0 fragment assembled with an octamer including fluorescently labelled H2B. Following native gel electrophoresis, the fate of H2B was monitored by fluorescent scanning of the gels. In some cases the signal moves to a location consistent with repositioning of nucleosomes on the donor DNA fragment. In others, transfer to the 0W0 acceptor DNA which has a distinct mobility, could be detected. Fun30 was observed to cause dimer exchange even in circumstances where repositioning was inefficient. (A) The donor nucleosome has an asymmetric linker DNA of 54 bp on one end and 18 bp on the other (54A18). (B) The donor nucleosome has 54bp linker DNA on one end and 0 bp on the other (54A0). (C) The donor nucleosome has 54 bp linkers on either side (54A54).

3.2.6. Fun30 can catalyze the transfer histone octamers *in trans*

A second remodelling mechanism is the transfer of an intact histone octamer from a nucleosome to a nucleosome-free region of the DNA (Lorch et al. 1999). This octamer transfer activity by chromatin remodelling proteins requires ATP hydrolysis. Since some chromatin-remodelling proteins including the SWI/SNF and RSC complexes possess octamer transfer activity, we wanted to know whether the yeast Fun30 also has the ability to transfer octamers *in vitro* to another fragment of DNA. To do this, we tested the ability of Fun30 to transfer octamers from short oligonucleosomes (SON) to a radio-labelled "GUB" naked acceptor DNA fragment.

This radio-labelled "GUB" DNA template was incubated with about 10 ng of donor SON in the presence of either SWI/SNF or Fun30 with or without ATP. Following incubation with remodelling enzymes assembly of nucleosomes onto the GUB fragment was assessed by native PAGE followed by radiography. The purified Fun30 complex like the SWI/SNF complex (Fig. 3.7, lanes 3-4), was able to transfer histone octamers from the cold donor SONs to the radio-labelled DNA template in an ATP-dependant manner (Fig. 3.7, lanes 5-6) generating slower migrating band with the same mobility as a mononucleosome (Fig. 3.7, lane 1). The percentage of labelled acceptor DNA bound by nucleosomes in transfer reaction was similar between the SWI/SNF complex and the Fun30 protein. These data demonstrate that Fun30 has the ability to transfer octamers *in trans* in an ATP-dependant manner. Moreover, the octamer transfer abilities of Fun30 and SWI/SNF are comparable.

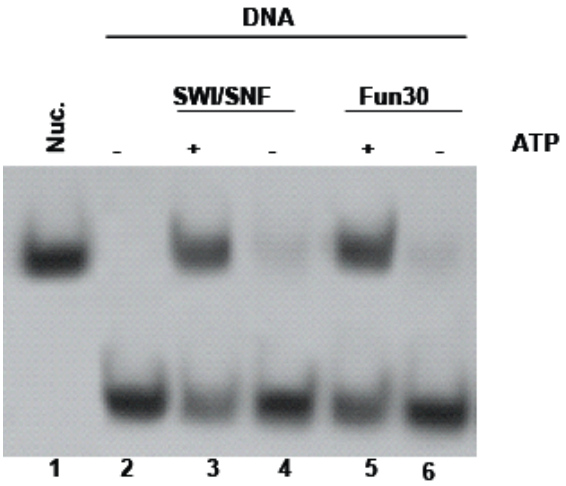

Figure 3.7. Fun30 has activity in octamer transfer. Fun30 (30nM) was incubated with 10ng radiolabelled 187bp GUB DNA fragment and 10ng on unlabelled HeLa small oligonucleosomes in the presence or absence of ATP as indicated and incubated at 30°C for 2 hours. Transfer of histone octamers from the HeLa DNA onto the GUB fragment can be detected as a shift in the mobility of the fragment following 4% native polyacrylamide gel electrophoresis and autoradiography. Lane 1 shows mononucleosomes assembled on the GUB DNA fragment. In the presence of Fun30 and ATP approximately 50% of the radiolablled DNA is assembled into nucleosomes.

3.2.7. Fun30 does not exhibit specificity for ubiquitinylated HeLa histones

In addition to the N-terminal to the region sharing homology with Snf2-related ATPases, Fun30 shares weak homology with conserved CUE and HHH domains (Fig. 3.9A) (Neves-Costa et al., 2009). CUE motifs in other proteins have been observed to interact with ubiquitin (Shih et al., 2003). We consulted with Karl Hoffman who is an expert on ubiquitin binding motifs. In his opinion, although the Fun30 CUE motif differs from related domains in well characterized ubiquitin binding proteins (An alignment of the CUE domain in Fun30 with other CUE domains is shown in the Appendix, Figure 8.2), this region is conserved in closely related yeast species, suggesting that it assumes a CUE/UBA fold and raising the possibility that it might bind ubiquitin.

Interestingly, we found that under low stringent TAP purification conditions that Fun30 is associated with a protein with the mobility anticipated for a ubiquitinylated histone (Fig. 3.8A-B). In order to investigate whether Fun30 preferentially binds to ubiquitinylated chromatin gel shifts were performed using HeLa oligonucleosomes. The proteins in the native gel were transferred to a nitrocellulose membrane and subject to Western blotting with and anti-ubiquitin antibody (Fig. 3.9B). Although Fun30 was capable of binding ubiquitinylated chromatin, no difference in the interaction of Fun30 with ubiquitinylated chromatin was observed in comparison to total H2B (Fig. 3.9C). This suggests that Fun30 does not have a preference in binding to ubiquitinylated histones. We also investigated whether there was a preference for transfer of ubiquitinylated histone dimers. To do this a dimer exchange assay similar to that shown in Fig. 3.6 but using HeLa donor chromatin

was performed. No preference for transfer of Ubiquitinylated dimers was observed (see Fig. 8.2 in Appendix).

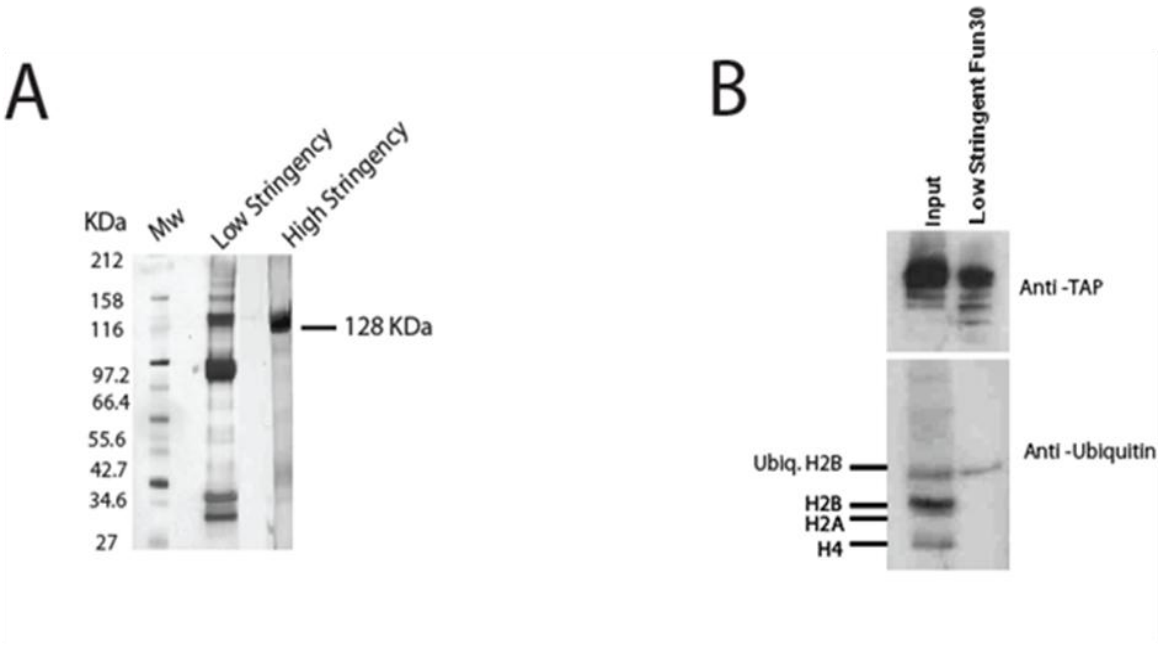

Figure 3.8. Fun30 recognize and interacts with ubiquitylated proteins. (A) Silver staining of the TAP purification of Fun30 under low stringent conditions, where interaction with histonal proteins could be detected. (B) Western blot analysis of low stringent purified Fun30 with anti-ubiquitin antibody to detect the presence of ubiquitylated proteins and anti-TAP antibody to detect Fun30. The 128 KDa band represent the Fun30 in both gel and blot. The input in the western blotting contains TAP-Fun30 and purified HeLa oligonucleosomes which contains modified histones.

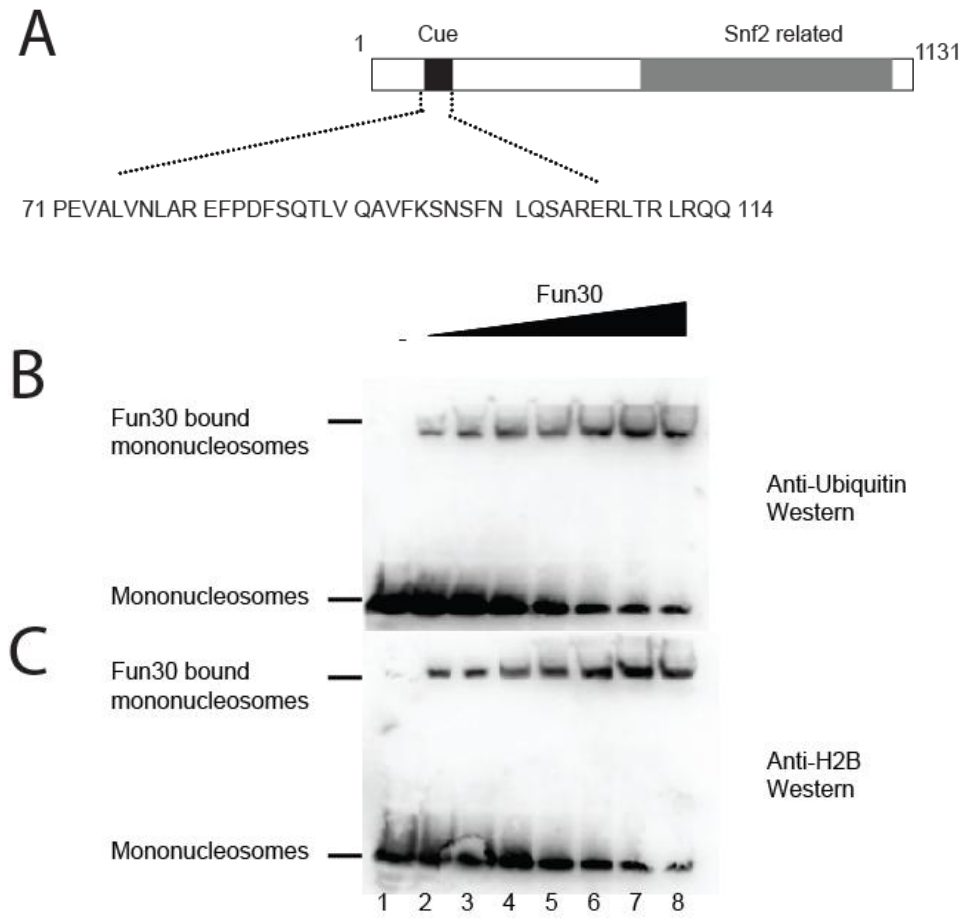

Figure 3.9. Fun30 contains a CUE motif, but no specific interaction with ubiquitinylated histones can be detected. (A) Schematic representation of CUE domain location within Fun30. A native gel in which ~ 200nM of HeLa mononucleosomes were incubated with increased quantities of Fun30 (~5-320 nM, lanes 2-8) was transferred to a PVDF membrane. (B) The transfer of ubiquitinylated histones was monitored by Western blotting. (C) Western blotting to detect the transfer of total H2B. No difference in the efficiency with which ubiquitinylated or total H2B is transferred could be detected.

3.2.8. Fun30 has low specificity towards H2AZ

Fun30 has more sequence homology with the Swr1, Ino80 and EP400 remodelling enzymes. Of them Swr1 has been shown to direct incorporation of the histone variant H2AZ. To investigate whether Fun30 also exhibit specificity towards H2AZ we compared the ability of Fun30 to transfer H2B and H2AZ dimers from HeLa donor chromatin to a tetrasome acceptor. Transfer of H2AZ was found to be slightly less efficient than H2B transfer (Fig. 3.10A and B). Quantification of this effect revealed a small but significant bias against the transfer of H2AZ in comparison to H2B (Fig. 3.11). This might be due to inefficient removal of H2AZ dimers from chromatin. Another possibility is that differences in the inherent stability of H2AZ in combination with other histone variants favour the reassociation of H2AZ with HeLa chromatin rather than transfer to the tetramer acceptor as reported in Jin et al., 2009 that different combinations of variants have different distributions, consistent with distinct roles for histone variants in the modulation of gene expression

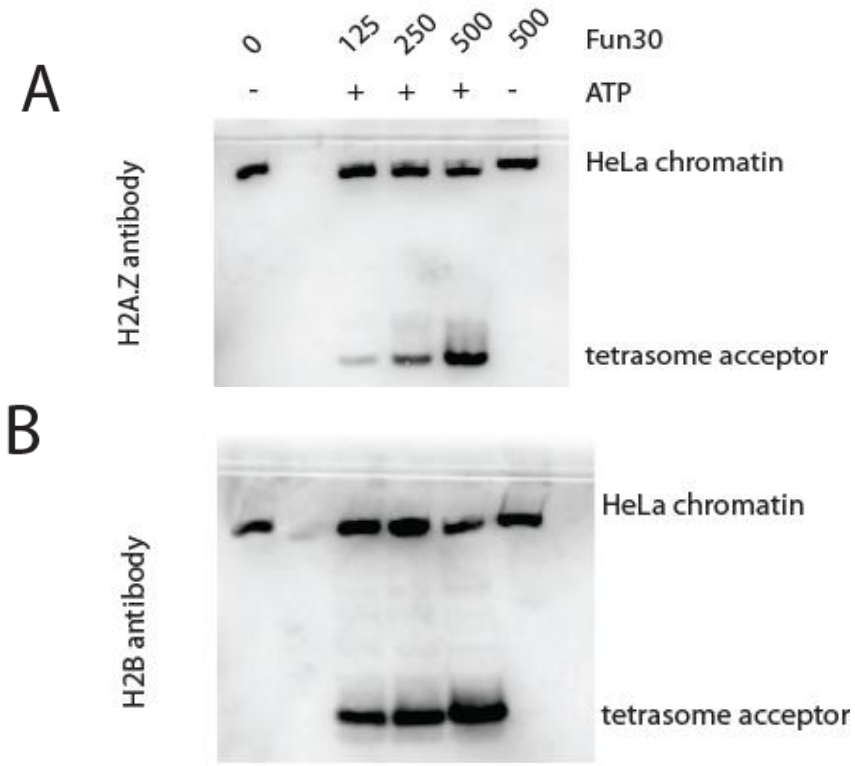

Figure 3.10. Fun30 transfers H2AZ/H2B and H2A/H2B dimers. (A). The transfer of H2AZ was monitored by Western blotting. (B). Western blotting to detect the transfer of total H2B. No difference in the efficiency with which H2AZ or total H2B is transferred could be detected. A native gel in which ~ 200nM of HeLa mononucleosomes were incubated with increased quantities of Fun30 (~125-500) nM, lanes 3-6) was transferred to a PVDF membrane. The transfer of H2AZ and transfer of total H2B histones were monitored by Western blotting.

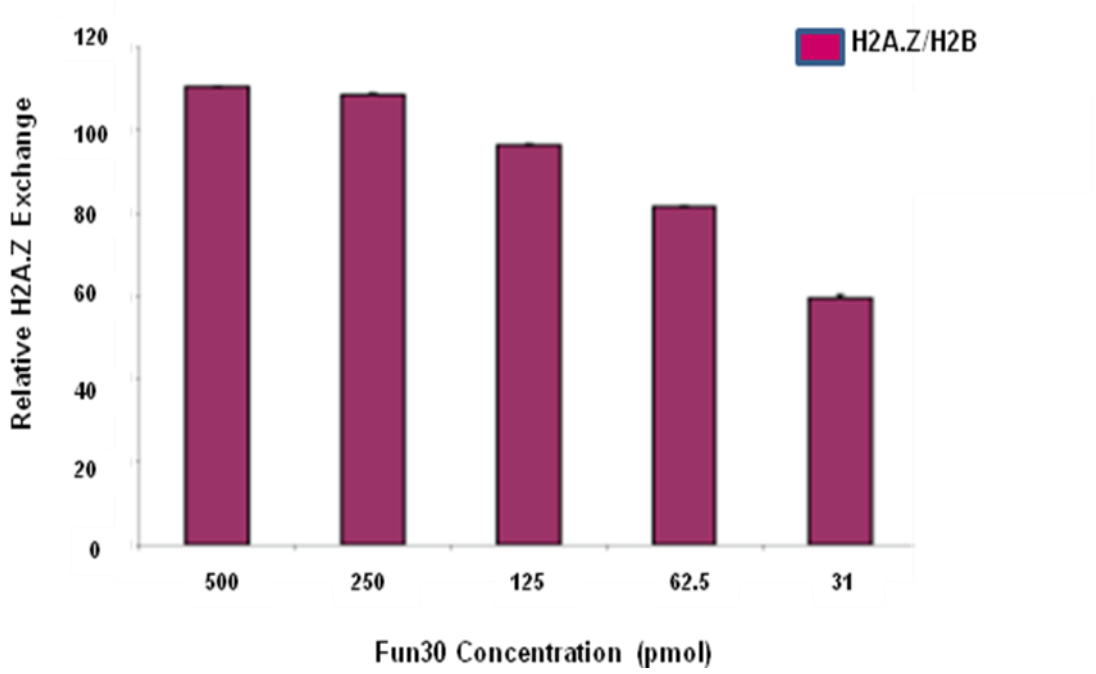

Figure 3.11. Fun30 has small but significant bias against the transfer of H2AZ in comparison to H2B. Quantification of H2AZ transfers efficiency by Fun30. The graph shows the mean value of the proportion of H2AZ transferred relative to H2B.

3.3. Discussion

The *Saccharomyces cerevisiae* Fun30 protein has been purified and found to exist predominantly as a homodimer. The silver staining of our TAP-purified Fun30 reveals a single major band with mobility consistent with that of Fun30. It is likely, however, that weakly interacting proteins would dissociate under the conditions used. Purification of Fun30 under low stringency conditions revealed some targets of substochiometric interactions that might be of functional significance such as H2A, H2B, translation elongation factors some ubiquitin proteases (see Table 8.1 in Appendix).

The observation that Fun30 elutes from gel filtration columns in a volume corresponding to a mass of 250 KDa could be interpreted as indicating the presence of a stable dimer. However, separately prepared TAP and His tagged Fun30 preparations were found to interact with each other *in vitro*. As a result, we favour the existence of a rapid equilibrium between monomeric and dimeric forms in solution. Quantitative analysis of Fun30 binding to nucleosomes suggest that the interaction is co-operative (Awad et al., 2010). This co-operativity could result from the stabilization of the dimeric form on nucleosomes. All together, these data provide strong evidence that the native Fun30 protein exists as a homodimer of 250 KDa. This been reported only for the ATP-dependent chromatin assembly factor (ACF). It has been shown that ACF consists of a heterotetramer of two ISWI (119 KDa) and two Acf1 (170 KDa) subunits with an apparent molecular mass of 690–730 KDa (Strohner et al., 2005). Recently, it has been reported that ACF maintains nucleosome spacing by constantly moving a nucleosome towards the longer flanking DNA faster than the shorter flanking DNA (Racki et al., 2009). This nucleosome movement depends cooperatively on two ACF molecules, indicating that ACF

functions as a dimer of ATPases (Racki et al., 2009). The lack of interacting proteins in addition to Fun30 fails to provide new leads into the function of this protein.

It is well known that ATP-dependent chromatin-remodelers utilize a variety of mechanisms to alter/disrupt the nucleosome structure and increase nucleosomal DNA accessibility (Racki and Narlikar, 2008). One of these mechanisms involves the movement or sliding nucleosomes *in cis*. Several chromatin remodelling complexes have been shown to possess sliding activities, although the outcome of the nucleosome sliding differs between some of these complexes. For example, whereas the ISWI protein has been shown to preferentially slide mononucleosomes positioned in the centre of DNA fragments toward the ends (Clapier et al., 2002; Hamiche et al., 2001; Dang et al., 2006), the CHRAC complex triggers the converse reaction and is able to move a nucleosome from one end to a more central position of a DNA fragment (Langst and Becker, 1999).

We have found that the Fun30 enzyme results in alterations to chromatin structure using a range of different assays. These included the generation increases in restriction enzyme accessibility, the transfer and repositioning of nucleosomes. However, Fun30 was found to be especially proficient in catalysing the exchange of histone dimers. Indeed on 54A0 and 54A54 templates Fun30 was able to catalyse the exchange of histone dimmers while no nucleosome repositioning was apparent (Fig. 3.6). This is consistent with the fact that based on sequence homology Fun30 is most closely related to the Swr1 and Ino80 proteins (Flaus et al., 2006) which have been reported to have activity in histone exchange (Mizuguchi et al., 2004; Papamichos-Chronakis et al., 2006). The observation that Fun30 is relatively inefficient in

repositioning nucleosomes supports previous work that suggested the mechanisms for dimer exchange and nucleosomes sliding are distinct (Ferreira et al., 2007).

It is been demonstrated that CUE domains bind directly to ubiquitin and represent an evolutionarily conserved ubiquitin-binding domain that mediates intramolecular monoubiquitylation (Ponting et al., 2000). As Fun30 contains a CUE motif that can potentially interact with ubiquitin, we sought to identify the possibility of interaction between Fun30 and any ubiquitylated proteins that may provide an insight into Fun30 function. The Human homolog of Fun30 protein (SMARCAD1) is annotated as containing CUE motifs that may be involved in recognizing ubiquitin (N-terminal to the Snf2 related region). The detection of proteins with the mobility expected for ubiquitylated histones associated with Fun30 purified under low stringent conditions raised the possibility this enzyme has specificity for ubiquitinylated histones. However, we could obtain no evidence for specific binding of Fun30 to ubiquitinylated histones (Fig. 3.9), or the ability to exchange ubiquitinylated histones (Fig. 8.1 in Appendix). A caveat to this experiment is that HeLa cells were used as a source of chromatin and the possibility remains that there is specificity for a feature of yeast chromatin we may have missed. This seems unlikely as ubiquitin peptides were not identified by mass spectrometry among the proteins interacting with Fun30 under low stringency conditions (See appendix Table 8.1). It is possible that the divergent CUE domains present in yeast Fun30 proteins are adapted to perform a different function.

The Swr1 complex exhibits specificity in histone exchange directing the incorporation of the histone variant Htz1 (Mizuguchi et al., 2004). This raised the possibility that Fun30 also has specificity in directing exchange of specific histone

subtypes; however, in our preliminary studies we obtained no evidence for this. Instead a slight preference for the transfer of non H2AZ containing nucleosomes was observed. This is reminiscent of the specificity of the ino80 complex for H2A/H2B dimers. However, the level of specificity we detected was lower (approximately 2-fold) and we are unsure whether this has physiological relevance. Some support for this is provided by previously reported genetic and physical interactions between Fun30 and both htz1 and components of the Swr1 complex (Krogan et al., 2003). How then could an enzyme with low specificity for this variant act to influence its function? One possibility is that the preference of Fun30 for binding nucleosomes bearing linker DNA directs the enzyme towards nucleosomes adjacent to nucleosome free regions. Htz1 is enriched in these regions (Jin et al., 2009; Hartley and Madhani, 2009) and it has previously been proposed that enzymes directing H2AZ incorporation might be directed to regions of exposed DNA (Hartley and Madhani, 2009). In this way non-specific histone dimer exchange especially out with S-phase could potentially contribute to the enrichment of Fun30 adjacent to nucleosome free regions.

3.4. Conclusions and perspectives

Here we report the purification of Fun30 principally as a homodimer with a molecular weight of about 250 KDa. Biochemical characterization of this complex reveals that it has ATPase activity stimulated by both DNA and chromatin. Consistent with this, it also binds to both DNA and chromatin. The Fun30 complex also exhibits activity in ATP-dependent chromatin remodelling assays. Interestingly, its activity in histone dimer exchange is high relative to the ability to reposition nucleosomes. Fun30 also possesses a weakly conserved CUE motif suggesting that it may interact specifically with ubiquitinylated proteins. However, *in vitro* Fun30 was found to have no specificity in its interaction with ubiquitinylated histones and little specificity for the histone variant H2AZ. It would be interesting to investigate the effect of CUE motif or ATPase domain deletions on the activity of Fun30.

Ch. IV: *FUN30* deletion results in cell cycle arrest at the G1-S transition

4.1. Introduction

Although Fun30 and its homologs are conserved in most eukaryotes, very little attention has been focused on the functions of these proteins. Subsequently a *FUN30* delete strain was created, but this was found not to affect chromosome stability. The deletion strain was however reported to be temperature sensitive (Clark et al., 1992). They have also been reported to be resistant to ultraviolet (UV) radiation (Barton and Kaback, 1994). Conversely, it has recently been shown that deletion of *FUN30* leads to sensitivity to the topoisomerase I poison camptothecin and to severe cell cycle progression defects when the Orc5 subunit is mutated (Neves-Costa et al., 2009). In addition to the small number of studies investigating the function of Fun30 there is a considerable volume of information relating the function of Fun30 to a range of cellular processes based on genome scale studies. Many of the reported interacting partners with Fun30 were proteins involved in cell cycle regulation, DNA replication and repair mechanisms. A summary of some of these findings is presented in table 8.2 in the appendix section, but some of the most interesting targets that have some relevance to this chapter will be mentioned below.

Several previous studies reported the identification of interaction partners for a significant numbers of yeast proteins. In the case of Fun30 these have resulted in the identification of 10 affinity capture interactions and 2 biochemical activity interactions (Krogan et al., 2003; Ubersax et al., 2003; Reinders et al., 2006; Collins et al., 2007; Tong et al., 2004). Tel1 is a protein kinase involved in telomere length regulation and contributes to cell cycle checkpoint control in response to DNA

damage (Gavin et al., 2002) was one of the proteins that physically interact with Fun30 and was affinity purified and analyzed by mass spectrometry. On the other hand, Cdc28 and CLB2 which are also involved in cell cycle progression were found to have biochemical activity interaction with Fun30 (Ubersax et al., 2003).

Ouspenski et al., 1999 performed a genetic screen to identify genes which when overexpressed resulted in high frequency chromosome loss. This screen resulted in the identification of 30 genes one of which is Fun30. It is quite interesting that this screen also recovered mutations in Ran GTPase system which plays a critical role in nucleocytoplasmic transport and has been implicated in the maintenance of nuclear structure and cell cycle control (Hughes et al., 1998). In total it has been reported that there are 34 negative genetic interactions, 8 positive genetic interactions, 1 phenotypic enhancement and 9 synthetic lethality interactions with Fun30 (see Table 8.2.). Among the targets that have genetic interaction with Fun30 are Arp 6 and 8 which are nuclear actin-related protein involved in chromatin remodelling and component of chromatin-remodelling enzyme complexes such as Ino80 (Hannum et al., 2009; Krogan et al., 2003).

In the case of Fun30 several interacting partners of special interest include Htz1, Orc2/3/5 and VPS71/72 which were reported to have synthetic lethality with Fun30 (see Appendix Table 8.2). These targets are of some significance to this thesis as they are involved in the process of DNA replication and DNA repair or the mechanism of chromatin remodelling. For example, Origin Recognition Complex subunits 2,3 and 5 directs DNA replication by binding to replication origins and is also involved in transcriptional silencing and is reported to be phosphorylated by Cdc28 (Suter et al., 2004; Neves-Costa et al., 2009). Conversely, Htz the yeast

histone variant exchanged for histone H2A in nucleosomes by the SWR1 complex is involved in transcriptional regulation through prevention of the spread of silent heterochromatin (Krogan et al., 2003). As for VPS71/72 they are nucleosome-binding components of the SWR1 complex (Krogan et al., 2003). Rad24 was the only partner that has phenotypic enhancement with Fun30. This is quite interesting as Rad24 is a checkpoint protein involved in the activation of the DNA damage and meiotic checkpoints (Beltrao et al., 2009).

An alternate means to identify functional interactions involves the use of a 2 hybrid screen. This has the advantage that protein interactions are monitored within living cells. However, 2 hybrid screens are also known to be capable of generating both false positive and false negative results. In the case of Fun30 only one two hybrid interaction partner was identified, LSM1 (Like Sm) protein. Lsm1 forms heteroheptameric complex (with Lsm2p, Lsm3p, Lsm4p, Lsm5p, Lsm6p, and Lsm7p) involved in degradation of cytoplasmic mRNAs (Fromont-Racine et al., 2000). Therefore, based on the evidence summarised earlier we sought to investigate the effects of deleting the *FUN30* gene with a special emphasis in examining the previously reported links to DNA damaging reagents and involvement in the cell cycle.

4.2. Results

4.2.1. *fun30* null mutant confers resistance to some DNA damaging agents and has sensitivity towards other compounds

To explore the physiological functions of *FUN30,* we investigated the phenotypes of the *fun30* null mutant (*fun30*Δ strain). First we investigated the involvement of *FUN30* in the functions of metabolic pathways. The strain was propagated on a variety of different carbon sources (raffinose, lactose and galactose). Serial dilutions of cells from wild-type, snf2, and *fun30* null mutant strains were plated on YPD and plates, , 20% galactose and raffinose and 20% lactose We found that the *fun30*Δ strain grows on rich media with a growth rate comparable to wt strains (Fig. 4.2.1A).

It has been suggested that *FUN30* is involved in chromosome stability and potentially DNA repair based on the pattern of genomic instability (Ouspenski et al., 1999). Thus, we investigated growth under conditions that could highlight the role of *FUN30* in the maintenance of DNA structure or DNA repair. To do this, the wild-type (wt), *snf2Δ*, and *fun30*Δ strains in the same genetic background were spotted onto YPD plates after their OD_{600} reached 1.5, and then different treatments to induce DNA damage. We first investigated resistance/sensitivity to UV irradiation. UV causes crosslinking between adjacent cytosine and thymine bases resulting in pyrimidine dimers which consequently inhibit polymerases and arrest replication. While the wild-type and *snf2Δ* strains are slightly more sensitive to even low doses of UV irradiation, the *fun30*Δ yeast cells confer resistance and grew well even at the highest UV exposure time interval (Fig. 4.2.1B). This supports a previous report of UV resistance in the absence of *FUN30* (Barton and Kaback, 1994). Next we investigated resistance to ionizing radiation (IR) using a similar approach. It is well known that IR causes breaks in DNA strands, which are particularly hazardous to the

cell because they can lead to genome rearrangements. We found that the cells of the *fun30*Δ strain were more resistant to increasing amounts of radiation. Taken together these observations suggested an involvement of *FUN30* in DNA repair.

Next, we investigated growth in the presence of other compounds that are known to cause DNA damage. We detected no significant variation between the wt and *fun30Δ* when hydroxyurea (HU) or Methyl methanesulfonate (MMS) were added to the media. HU reduces the production of deoxyribonucleotides resulting in defective DNA replication. On the other hand, MMS methylates DNA causing double-stranded DNA breaks which are thought to cause replication forks to stall (Lundin et al., 2005). These findings suggested that there is no defect in the repair mechanism responsible for the response to HU or MMS when *FUN30* is deleted. Furthermore, we found that *fun30* null mutant cells were slightly sensitive to caffeine than Wt (Fig. 4.2.1). It has been reported that caffeine has the capacity to disrupt the S-phase checkpoint in the fission yeast *Schizosaccharomyces pombe* (Wang et al, 1999). This finding indicated that *FUN30* may be important for the maintenance of cell viability during S phase arrest. These data together suggest the possible role of *FUN30* in cell cycle, DNA damage repair and/or processing.

Addition of 6-Azauracil (6AU) to media results in a reduction in intracellular GTP levels. This reduction in GTP levels is not itself lethal, but can prevent yeast growth when combined with mutations that affect transcriptional elongation (Tansey, 2006). 6AU sensitivity thus can be used as a crude assay to test for mutations that affect transcriptional elongation. When 6-AU was added to the plates, we observed that the *fun30* null mutant cells grew better than wild type cells (Fig. 4.2.1C). This

observation suggested that *FUN30* may have functions related to the elongation of transcription. We also observed increased sensitivity of the *fun30*Δ strain to ethidium bromide (Fig. 4.2.1C).

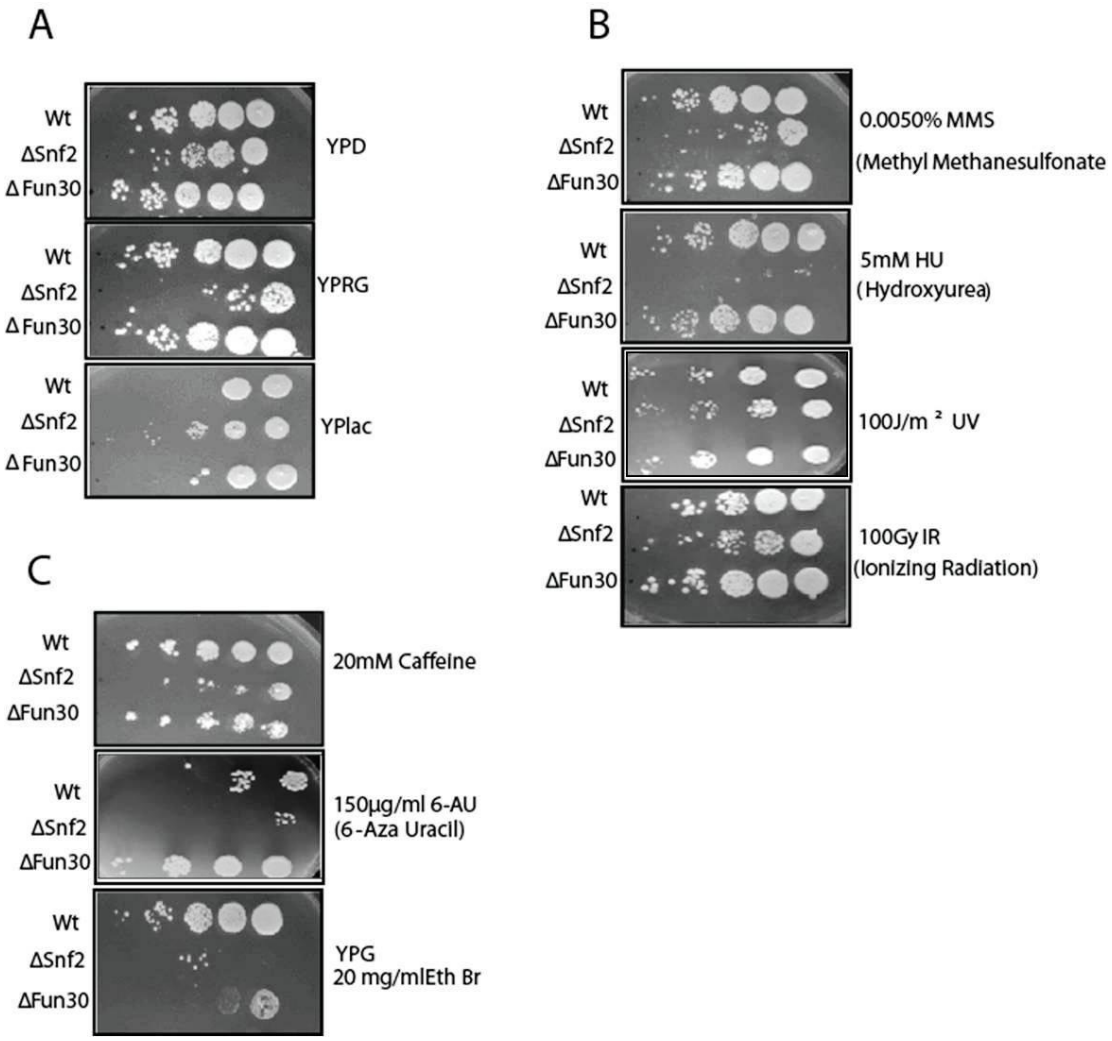

Figure 4.2.1. Analysis of the fun30 null mutant phenotypes shows that this mutant is resistant to DNA irradiation but sensitive to Ethidum bromide. (A) Serial dilutions of cells from wild-type, *snf2*, and *fun30* null mutant strains were plated on YPD and plates, , 20% galactose and raffinose and 20% lactose (B) The *fun30* null mutant and Wt growth was tested against MMS, HU,UV and IR. (C) Wt, *snf2*, and *fun30* null mutant strains growth against 6-AU and sensitive to YPG-Eth-Br and caffeine. The plates shown are representatives of at least two individual experiments.

4.2.2. *fun30* deletion results in a temperature dependent delay in G1/S phase

In the introduction strong links between *FUN30* and genes involved in progression through the cell cycle and DNA replication in particular were mentioned. To characterize the ability of *fun30* mutants to progress through the cell cycle, flow cytometry following propedium iodide staining was used to monitor the DNA content of cultures synchronized using α-factor. The separation of cells in G_1/S phase and G_2/M was based upon linear fluorescence intensity. Representative profiles are shown in following figures (Fig. 4.2.2-4.2.3 and Fig. 4.2.6). Following release from α-factor arrest, both wt and the *fun30*Δ strains initially consisted predominantly of cells with a 1C (left peak) DNA content (Fig. 4.2.2) indicative of the G1/S phases of the cell cycle. Over time the proportion of cells with a 2C (right) DNA content (G_2/M) increased steadily reaching a peak after approximately 100mins for the wt and 140min for the *fun30*Δ. The delay in the *fun30*Δ strains suggests there is a delay in the progression through these stages of the cell cycle.

It has previously been reported that deletion of *FUN30* results in temperature sensitivity. To investigate whether this temperature sensitivity occurs as a result of a defect in progression through the cell cycle, cultures were shifted to this temperature following release from α-factor arrest. At 37°C we observed that in *fun30*Δ the percentage of cells in G1/S phase stayed almost constant compared to wt (Fig. 4.2.3.), suggesting an arrest at this stage of cell cycle based on analysis on longer time points up to 200 min (data not shown).

Budding index was monitored as an independent measure of progression through anaphase. Consistent with the FACS analysis a delay in budding was observed (Fig. 4.2.3 B). This suggests that the delay to progression through the cell cycle observed at 30°C becomes more pronounced at 37°C.

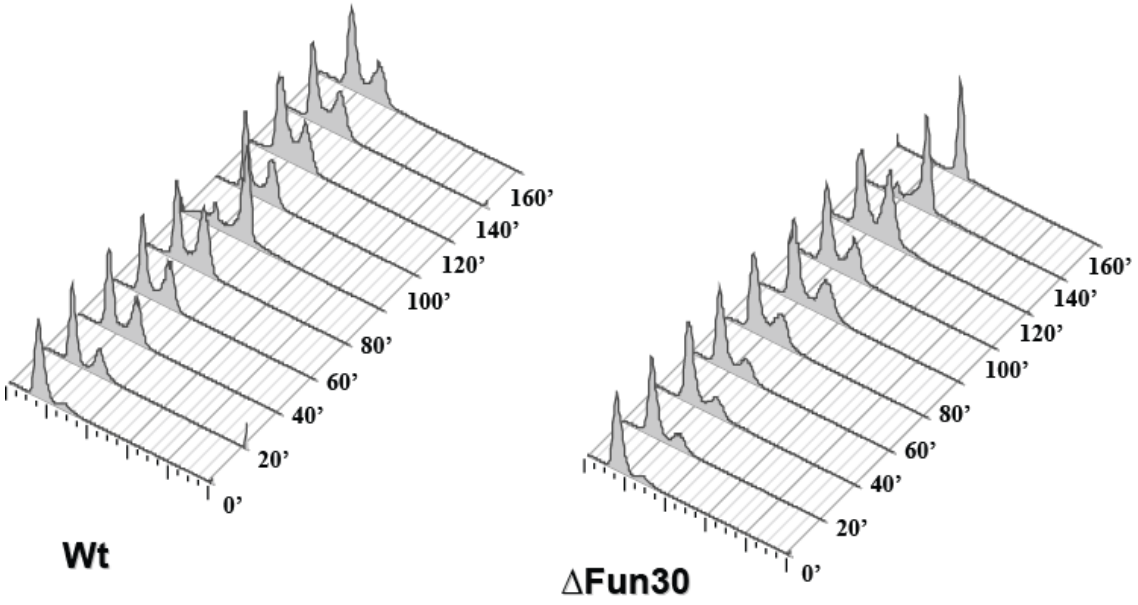

Figure 4.2.2. DNA content of the fun30 null mutant and the wt using PI at 25°C. Cells were synchronized in G1 by α-factor. After washing, the cells were resuspended in fresh medium and incubated at 25°C. Samples taken at intervals were analyzed for DNA content by FACScan.

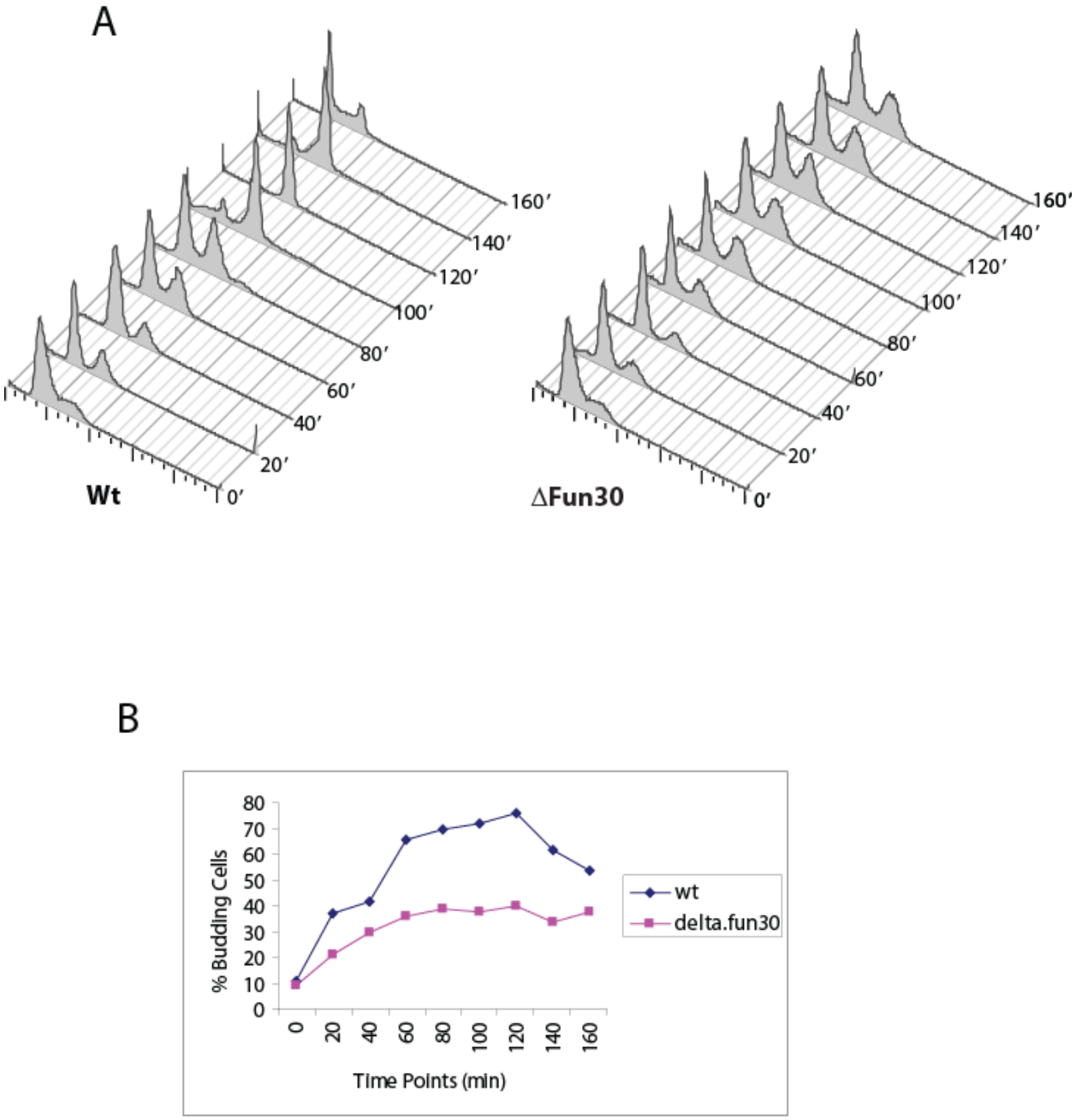

Figure 4.2.3. Non-permissive temperature induces a transient G1/S arrest in the fun30 null mutant. DNA content of the *fun30* null mutant and the Wt using PI at 37°C were measured. Cells were synchronized in G1 by α-factor. After washing, the cells were resuspended in fresh medium and incubated at 37°C. Samples taken at intervals were analyzed for DNA content by (A) FACScan and (B) Budding profiles.

4.2.3. Delayed progression to anaphase is also observed following ablation of *FUN30*

The use of conditional mutants is a powerful means of addressing the function of genes. However, one limitation of the classical *ts* approach is the uncertainty as to whether all function is lost under non permissive conditions. An alternative approach is to inducibly degrade proteins of interest. This can be achieved by fusing the gene of interest to Arg-DHFR^{ts} (MW= 140 Kb), a *ts* variant of dihydrofolate reductase–bearing N-terminal Arg residue (a destabilizing residue in the N-end rule) which only becomes exposed at 37°C as a result of misfolding of the DHFR ts protein. As a result proteins of interest are degraded at 37°C, but stable during growth at 25°C. We used this approach to construct a "heat-inducible Degron system" for *FUN30*.

To test for correct integration of the degron construct, primer pairs were generated that would distinguish between the parent strain and successful integrants. As shown in Fig. 4.2.4. PCR products diagnostic of the correct integration of the degron construct were detected. In order to test the functionality of the degron, the presence of Fun30 was next monitored by western blotting before and after induction of the degron (See Fig. 4.2.5A).Within 30 min the majority of Fun30 had been degraded.

As we found that deletion of *FUN30* slowed growth at 37°C we next investigated whether this was also the case following depletion of Fun30. Growth following activation of the degron was found to be reduced at 37°C (Fig. 4.2.5B). Next we investigated the consequences of depleting Fun30 on progression through the cell cycle at 37 °C. When DNA content was monitored by FACS a delay in accumulation of cells with 2C content was observed (Fig. 4.2.6). Similarly the proportion of budding cells was reduced following degron induction (Fig. 4.2.6). These observations are consistent with the deletion of *FUN30* causing an accumulation of cells in G1/S phase at the non-permissive temperature.

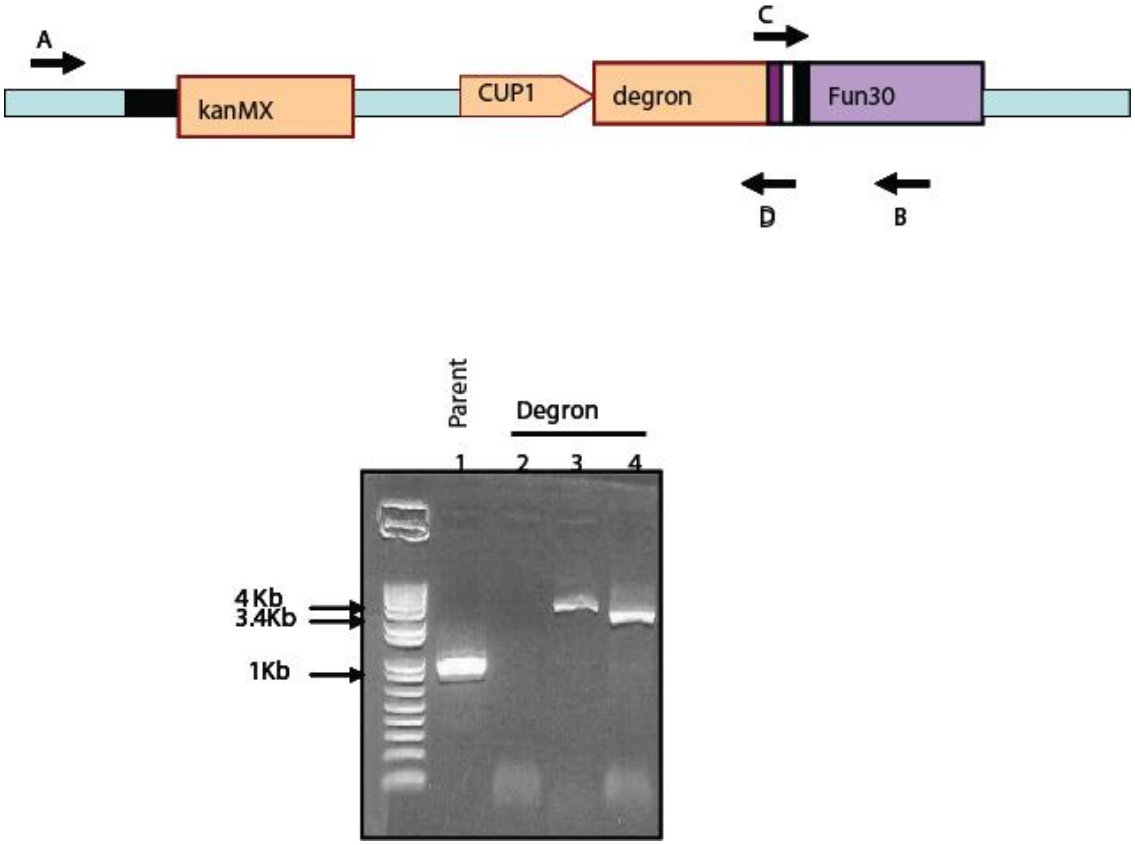

Figure.4.2.4. Selection of correctly integrated transformants. To test whether the degron cassette has integrated correctly, four primers (A-D) were used to check the integration as described in the text. The correct degron integration should yield the following bands: The parental strain A+B = 1 Kb (lane 1), C+D = 0Kb (lane2), A+B = 3.9 Kb (lane 3) and A+D = 3.4 Kb (lane 4).

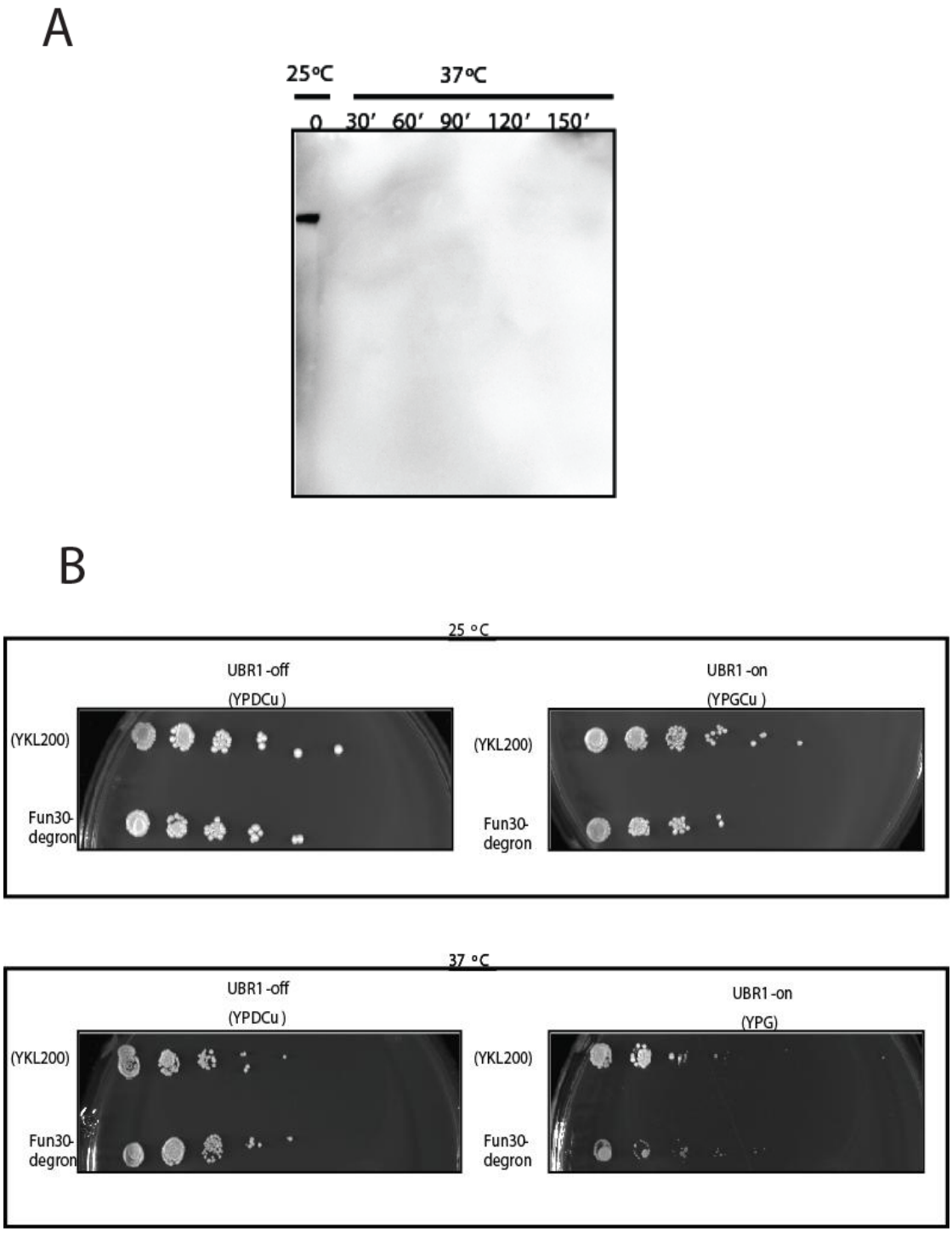

Figure. 4.2.5. Checking the degron viability at 37°C. (A) Fun30-degron was cultured at 25°C, then expression of Ubr1 was induced and cells were transferred to 37°C for the indicated time periods, and the Fun30-degron was detected using anti-myc. (B) The growth of the temperature sensitive degron is inhibited at 37°C in the presence of high levels of Ubr1. Serial dilutions of the cells were made for the parental strain (YKL2000) and the Fun30-degron, and then the cells were spotted onto plates under the indicated conditions and incubated for 2-3 days. Depletion of the target protein was rapid after fusion to the degron.

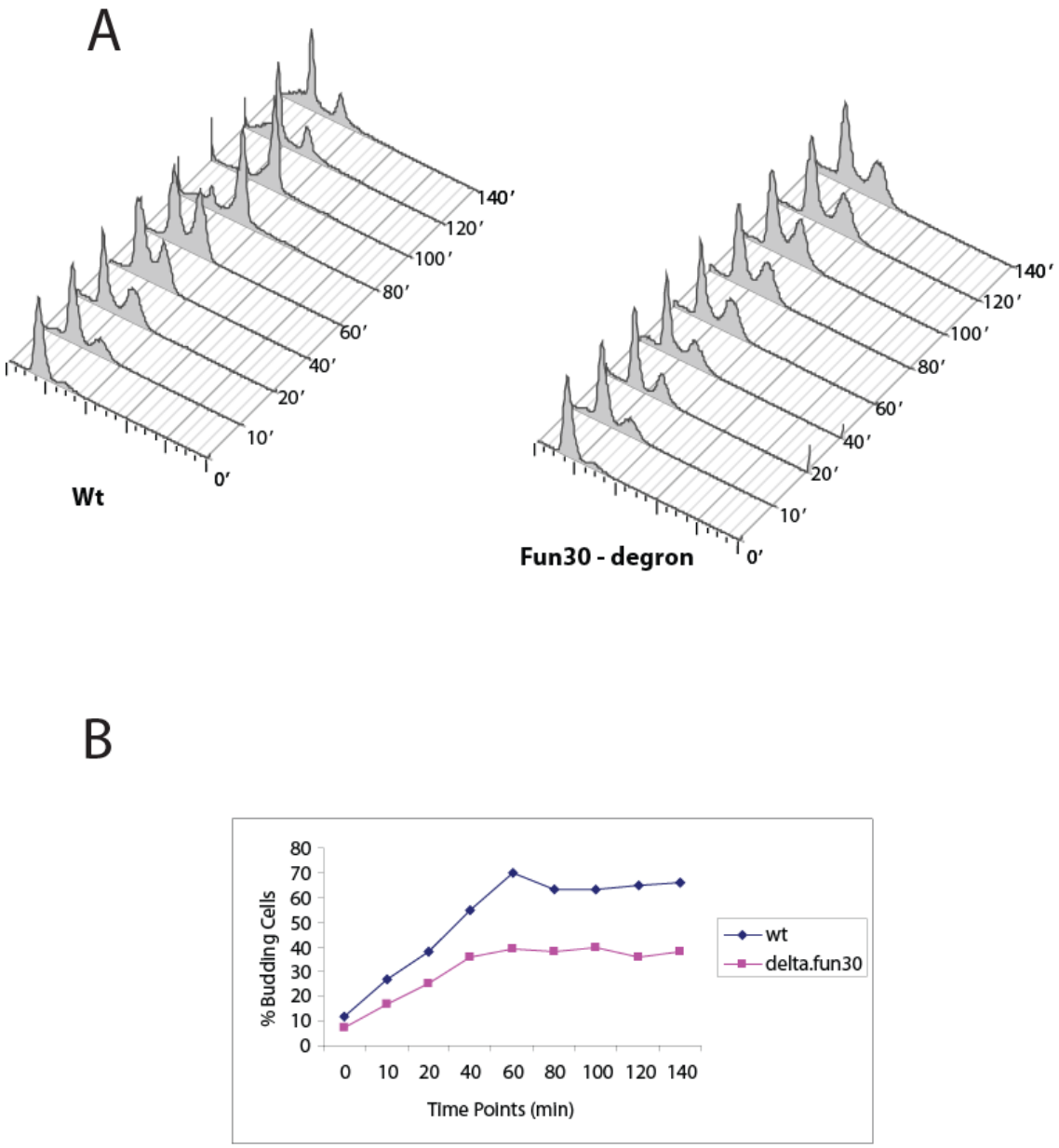

Figure 4.2.6. Depletion of Fun30 in the degron fused strain induces a transient G1/S arrest at 37°C. DNA content of the Fun30-degron and the Wt using PI at 37°C were measured. Cells were synchronized in G1 by α-factor at 25°C. After washing, the cells were resuspended in fresh medium and incubated at 37°C. Samples taken at intervals were analyzed for DNA content by (A) FACScan and (B) Budding profiles.

4.2.4. *fun30* deletion increases the expression of phosphorylated Rad53, Rad9 and Cdc28

Since the flow cytometry does not discriminate between the G1 and S phases of the cell cycle in the case of the yeast, we decided to examine the expression of several checkpoints marker proteins specific to these phases. It has been reported that activation of the DNA damage checkpoint in G1/S results in rapid phosphorylation of the yeast 53BP1 ortholog and checkpoint adaptor protein Rad9 and subsequent binding and recruitment of the checkpoint effecter kinase Rad53, which then transautophosphorylates and becomes active (Gilbert et al., 2001) see Fig. 4.2.7.

Moreover, it has been reported that Rad53 aids in maintaining stalled replication forks (Desany et al., 1998). We observed that the null mutant of *fun30* can result in increased activation of the phosphorylated forms of Rad 9 and Rad 53 further supporting the notion that *FUN30* deletion can result in cell cycle arrest in S-phase and suggest its involvement in replication related processes (Fig. 4.2.8). This observation suggested that under these conditions deletion of *FUN30* results in activation of the S phase checkpoint. Furthermore, we found that null mutant of *fun30* results in increase Cdc28 level (Fig. 4.2.8). Cdc28 is the master regulator of cell division in the budding yeast *Saccharomyces cerevisiae* its activity controls the timing of mitotic commitment, bud initiation, DNA replication, spindle formation, and chromosome separation (Mendenhall and Hodge, 1998). All these data together indicate that *FUN30* deletion causes cell cycle arrest during S- phase.

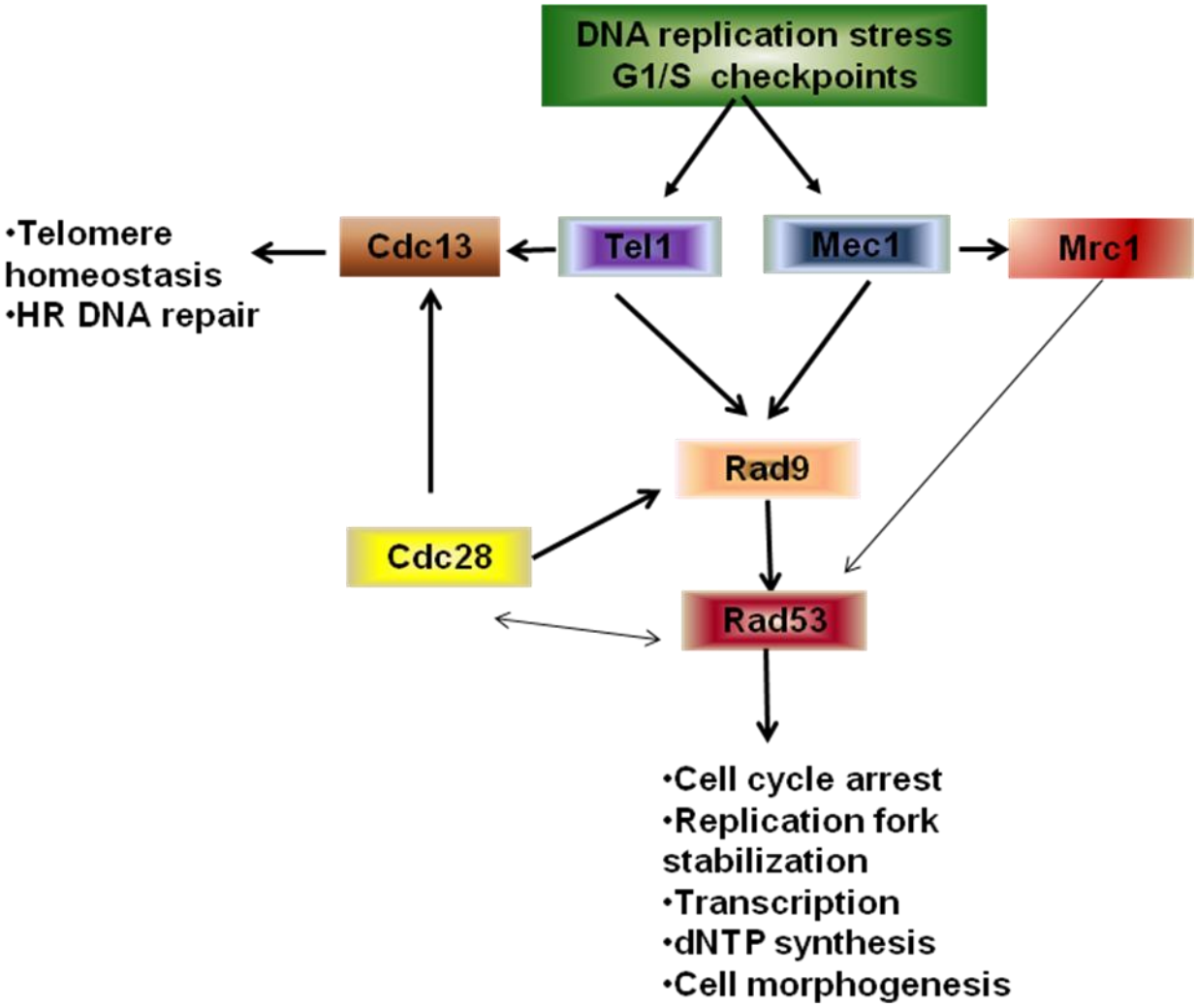

Figure.4.2.7. Schematic representation of Rad53 activation due to G1/S checkpoint response. DNA damage and replication stress are sensed by a number of proteins that activate the PIKKs Mec1 and Tel1. These kinases activate a signal transduction pathway consisting of the adaptor protein Rad 9 and the kinases Rad53and Cdc28. Cdc28 may phosphorylate Rad9 to boost the signalling cascade. Cdc28 also phosphorylates Rad53. Modified from Enserink and Kolodner, 2010.

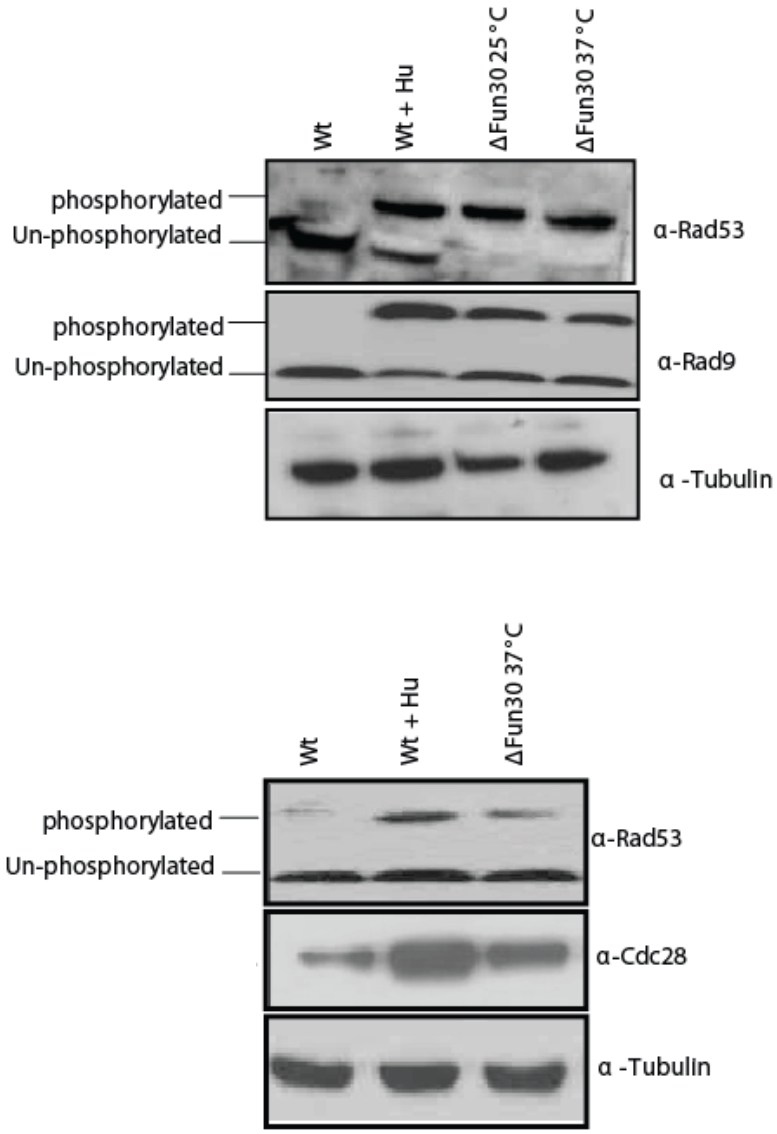

Figure 4.2.8. Increased Rad53, Rad9 and Cdc28 activity in response to fun30 null mutant. Samples were taken at the Log-phase and analyzed by western blotting. Wt strain were treated with 0.2 M HU for 3 h (HU), washed with the same volume of YPD to remove HU, and released from the HU block and 40µg of total protein was prepared from the indicated strains and analyzed by Western blotting using anti-Rad53, anti-Rad9 and anti-Cdc28 (Santa Cruz, USA)

4.2.5. *fun30* null mutation decreases the level of H3 K79 dimethylation

Recently, several histone modifications such as ubiquitination, acetylation, and methylation have been implicated in the DNA damage checkpoint and repair pathways. Thus we explored the levels of some of these modifications in a *fun30*Δ mutant strain. We observed a slight decrease in the level of H3K79 dimethylation and the ubiquitination of H2B and H4, with no effect on the K4 di/tri methylation or Htz levels (Fig. 4.2.9).

The slight decrease in the level of H3K4/K79 dimethylation we have detected is consistent with the report by Neves-Costa et al. (2009) that Fun30 plays a role in silencing as H3K79 methylation is also required for heterochromatin formation (Jones et al., 2008). Furthermore, it has been shown that histone H3 K79 methylation is important for repair of UV-induced DNA damage in *Saccharomyces cerevisiae*, acting through multiple repair pathways. It has been found that K79 methylation may be modulated in response to UV damage via a trans-histone regulatory pathway, and that distinct methylation states may provide a means of coordinating specific DNA repair and damage checkpoint pathways (Evans et al., 2008). Interestingly, one of the main findings in Schulze et al. 2009, that K79 methylation is cell cycle regulated and decorates the ORFs and intergenic regions, including the promoters, of genes expressed specifically in the G1/S phase of the cell cycle. Moreover, it is stated that levels of H3K79 methylation are low during G1/early S and are maintained throughout G2/M, when the genes are inactive. Thus, the depletion of K79 might occur as a result of an increased proportion of cells in the G1 S phase of the cell cycle in a *fun30* mutant.

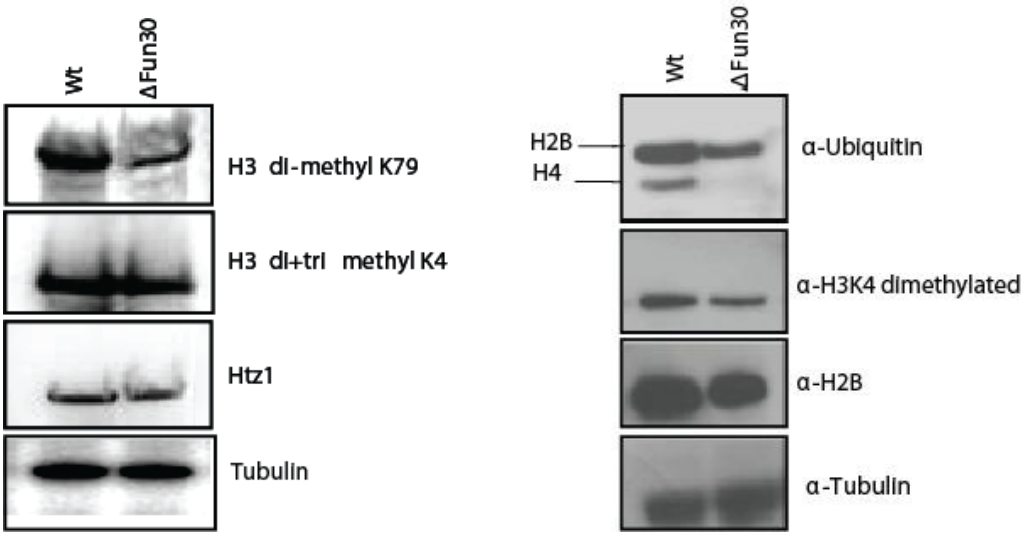

Figure 4.2.9. *fun30* null mutant affected the expression of H3 79 methylation and histone ubiquitylation. 40 µg of total protein from wild type (wt) and *fun30*Δ, probed with different histonal modifications antibodies and anti-tubulin to ensure equal loading was loaded into 4-12% SDS-PAGE and analyzed by Western blotting using antibodies against the indicated targets.

4.3. Discussion

In order to gain insight into the function of the Fun30 protein in the yeast, we investigated the phenotypes of a *fun30* null mutant strain. We found that a *fun30* null strain is resistant to UV irradiation and ionizing radiation. There are a number of different potential explanations for the resistance of the *fun30*Δ strain to ultraviolet and ionizing radiation. One possibility is that genes involved in the repair of ultraviolet/ionized-damaged DNA are activated in the *fun30*Δ as Fun30 might act as silencer to these genes. This is supported by the recent finding of Fun30 silencing ability by the Varga-Weisz lab (Neves-costa et al., 2010). On the other hand, it is been reported recently that yeast cells can die as result to the process of programmed cell death (Madeo et al., 2004). Thus, an alternate explanation could involve a role in the activation of genes that are anti-apoptotic and thus rescue cells from undergoing apoptosis. Another possibility is that defective progression through the cell cycle enables cells to contend with certain types of DNA damage.

Interestingly, *fun30*Δ cells also conferred resistance to growth on plates containing 6-AU, which leads to a reduction of intracellular GTP levels. The decline in GTP levels is not itself lethal, but can inhibit yeast growth when combined with mutations that affect transcriptional elongation. Thus it's possible that the *FUN30* deletion affects transcriptional elongation. Another link to transcriptional elongation is provided the decreased growth of the *fun30* knockout on ethidium bromide plates. Ethidium is known to affect the termination of transcription termination. The interactions between Fun30 and proteins involved in transcription such as Taf13, Rpo21, Rpc40 and Rpc34

(Collins et al., 2007) provide additional links to Fun30 involvement in transcription.

Flow cytometry analysis revealed that mutant *FUN30* cells under non-permissive temperature accumulated in the G1 or early stage of the S-phase of the cell cycle. This suggests that *FUN30* normally acts to ensure normal progress through S phase. Consistent with there being a defect in S phase in the absence of Fun30, we detected increased phosphorylation of Rad53 and Rad 9. This indicates partial activation of the S- phase checkpoint. The *fun30* null mutant cells also showed sensitivity towards caffeine. Caffeine acts as an inhibitor of the Tel1 and Mec1 kinases which are the upstream activators of Rad53 (Saiardi et al., 2005). These observations are all consistent with deletion of *FUN30* resulting in defects in DNA replication. Activation of the S phase checkpoint in the absence of *fun30* may be important to ensure that these defects are corrected before replication is completed. An additional link between Fun30 and the S-phase checkpoint is provided by the recent observation that Fun30 itself is phosphorylated by Mec1/Tel1, Rad53, and Dun1.This adds to a series of interactions that have been detected between Fun30 and proteins involved in cell cycle regulation. For example, Fun30 displays synthetic lethality with 3 subunits of the ORC, Mad3, Tel1 and Cdc28 complex which are reported to be important for genome integrity and replication maintenance (Suter et al., 2004; Neves-Costa et al., 2009). The strong links between Fun30 and DNA replication suggested that it would be of interest to study the role of Fun30 during the cell cycle in more detail.

4.4. Conclusions and perspectives

In an attempt to elucidate the *in vivo* function of *FUN30*, we found that the *fun30* null mutant is resistant to agents that induced DNA damage such as UV and IR. These data suggest that *FUN30* plays a role in DNA repair. Moreover, we have also shown that *fun30* null mutant cells are resistance to 6-AU suggesting it is also involved in the process of transcription.

Furthermore, *FUN30* deletion results in increased expression of Rad53, Rad9 under non permissive temperature and causes cells accumulation at the S-phase. These observations suggest that *FUN30* plays a role in normal progression through S-phase. All these data together, provide some insights into the function of this enigmatic protein, but at the same time they add more questions that remain unanswered. One interesting area to explore further would be to carefully elucidate if the cell cycle arrest due to *FUN30* deletion is resultant of errors in DNA damage repair or replication pathways.

Ch. V: Genome-Wide analysis indicates transient association of Fun30 with DNA replication origins during the S-phase of the cell

5.1. Introduction

A powerful approach for monitoring the association of DNA binding proteins with DNA is Chromatin Immunoprecipitation (ChIP). This involves the purification of cross-linked chromatin fragments associated with an epitope of interest. Following purification, the cross-links can be reversed and the DNA purified. In some of the original applications of ChIP antibodies were used to enrich for DNA fragments associated with histone modifications (Saleh et al., 2008; Huebert et al., 2006). In these cases the regions of DNA enriched were identified using quantitative PCR or slot blotting.

The development of genomic technologies provided an opportunity to extend the capability of ChIP from the study of individual genes to entire genomes. For example, if ChIP purified DNA is amplified and labeled with fluorescent dyes, it can be hybridized to DNA microarrays on which fragments derived from specific genomic locations are tiled at high density. The enrichment of fluorescent signal can then be used as a measure for occupancy at each location covered by the microarray.

Early applications of this approach, which is commonly referred to as ChIP-chip, include the identification of binding sites for transcription factors in the yeast *Saccharomyces cerevisiae* (Ren et al., 2000; Lieb et al., 2001; Iyer et al., 2001). Subsequently this has been expanded to include 106 transcription factors using a c-Myc tagging system (Lee et al., 2002).

At the level of chromatin remodelling and modification ChIP-chip technology has been applied to great effect. For instance, Nucleosome modifications across the yeast

genome were profiled using ChIP-chip technology to produce high-resolution genome-wide maps of histone acetylation and methylation that take into consideration the alterations in nucleosome occupancy at actively transcribed genes (Pokholok et al., 2005).

In addition ChIP-chip technology has been used to investigate the distribution of some key chromatin components over the whole genome. For instance, the study conducted by Li et al.(2005) used the genome-wide analysis to map the distribution of Htz1 (histone H2A variant) in the genome of yeast *Saccharomyces cerevisiae*. They showed that Htz1 was enriched in intergenic regions compared with coding regions, and that its occupancy is inversely proportional to transcription rate and the occupancy of RNA polymerase II (pol II) in adjacent genes (Li et al., 2005).

Additionally, ChIP-chip technology has been successfully applied to study a range of regulatory processes such as understanding the mechanism of Isw2 association with chromatin *in vivo* (Gelbart et al., 2005).

There are many microarray chips used to conduct ChIP-on-chip experiments the most commonly used are Affymetrix, Illumina, Agilent, and Roche Nimblgene. They can vary according to probe size, probe type, probe composition and array size (Royce et al., 2005; Buck and Lieb, 2004). The first sets of microarrays were designed to study gene expression profiles, and have limited applications in ChIP experiments because most of the interesting target proteins bind in the intergenic regions (Buck and Lieb, 2004).

Nowadays the arrays use probes as short as 25-mers (Affimetrix, 2010). Arrays can be divided into tiled and non-tiled DNA arrays. The non-tiled arrays use probes that have no fixed distances in the genome, whereas the tiled arrays uses probes that are selected within the genomic region (or even a whole genome) and divide them into

equal (or near equal) pieces called tiled paths (Royce et al., 2005, Buck and Lieb, 2004).

Early ChIP-on-Chip studies used microarray chips that contained about 13,000 spotted DNA segments representing all ORFs and intergenic regions from the yeast genome (Buck and Lieb, 2004). However, currently many companies offer whole-genome tiled yeast arrays with a resolution ranging from 5bp up to 250bp (all in all 3.2 million probes) (Buck and Lieb, 2004; Affimetrix, 2010) . In addition to the actual array, other equipment is necessary to run ChIP-on-chip experiments. This includes hybridization ovens, chip scanners, and software packages for subsequent numerical analysis of the raw data. Often, one company's microarrays cannot be analyzed by another company's processing hardware. Therefore, it is necessary to buy the array, with its associated workflow equipments (Royce et al., 2005, Buck and Lieb, 2004).

ChIP-on-chip experiments involve preparation of -DNA, followed by hybridizing the sample to a microarray for subsequent analysis. The major steps include: rapid fixation of cells to chemically cross-link DNA binding proteins to their genomic targets *in vivo*. This is followed by cell lysis, which releases the DNA-protein complexes followed by sonication to fragment the DNA. Immunoprecipitation (IP) with a specific antibody is then conducted to purify the protein-DNA fragments. Cross-links are then reversed to allow release of DNA fragments. These are then amplified, labeled, and hybridized onto customized microarrays. Binding is detected using a high resolution fluorescent scanner. Following normalization, signal can be aligned with annotated genome databases using custom ChIP Analytics software (Buck and Lieb, 2004).

More recently, the direct sequencing of DNA fragments has emerged as an alternative to the use of microarrays (Johnson et al., 2007). In this approach, short sequence reads are obtained from one or both ends of the immunopurified fragments and these can be used to identify where in the genome they originate from. The approach has the advantage that resolution is only limited by fragment size and the dynamic range can be very high. Disadvantages include the cost and access to a facility with a reasonable turnaround time. With relevance to the chromatin field Chip-seq has been successfully applied to the mapping of nucleosome positions (Schmid and Bucher, 2007). In this Chapter progress in adopting the ChIP-chip approach to investigate the function of the *FUN30* protein is reported on.

5.2. Results

5.2.1. Optimization of the ChIP-chip for Fun30

As antibodies for Fun30 are not available, we used a yeast strain in which Fun30 was fused to an epitope tag. In this case we chose to use the TAP-tag that has previously been used for ChIP (Hecht and Grunstein, 1999; Schaft et al., 2003). We first sought to optimize the conditions in order to guarantee that there is a Fun30 dependent enrichment of some loci over others. First we confirmed that the IP reaction was specific to pull down Fun30 by western blotting against anti-TAP antibody (Fig. 5.2.1.A). This also provided an opportunity to optimize the washing stringency, which was three washes wish ChIP washing buffer. Having established conditions for the efficient affinity purification of Fun30 bound chromatin we next wanted to measure the enrichment of Fun30 target DNA. A problem here was that no Fun30 targets had been identified. With the aim of identifying putative positive control genes, we decided to sequence some of the IP'd DNA. This was performed by subcloning fragments into plasmids, and preparing DNA from single transformed colonies. Initial sequencing reads identified DNA fragments derived from the Gir2, Ena1 and BUL1 genes (see Table 5.2.1). Oligos were designed so that PCR could be used to assess enrichment of these genes in decross-linked chromatin or genomic DNA as a control (Fig. 5.2.1.B). Gir2 and Bul1 were reproducibly found to be enriched in bound chromatin whereas several other loci were not.

250 ng of Fun30 enriched DNA are required for labeling and hybridization by the Nimblgene in house service. We could prepare 10 ng of Fun30 enriched DNA from $2X10^6$ cells). This was then amplified using the GenomePlex® Whole Genome Amplification (WGA) kit from Sigma approximately 25-fold. In general the WGA Kit utilizes a proprietary technology based on random fragmentation of genomic

DNA and conversion of the resulting small fragments to PCR-amplifiable library molecules flanked by universal priming sites. WGA is achieved by PCR amplification of the library molecules using universal oligonucleotide primers. Amplification requires only a small amount of starting material (10 ng of DNA), which after PCR produces a yield of 5 - 10 µg per reaction. This method enabled us to prepare DNA on the scale required for hybridization (Fig. 5.2.1.C).

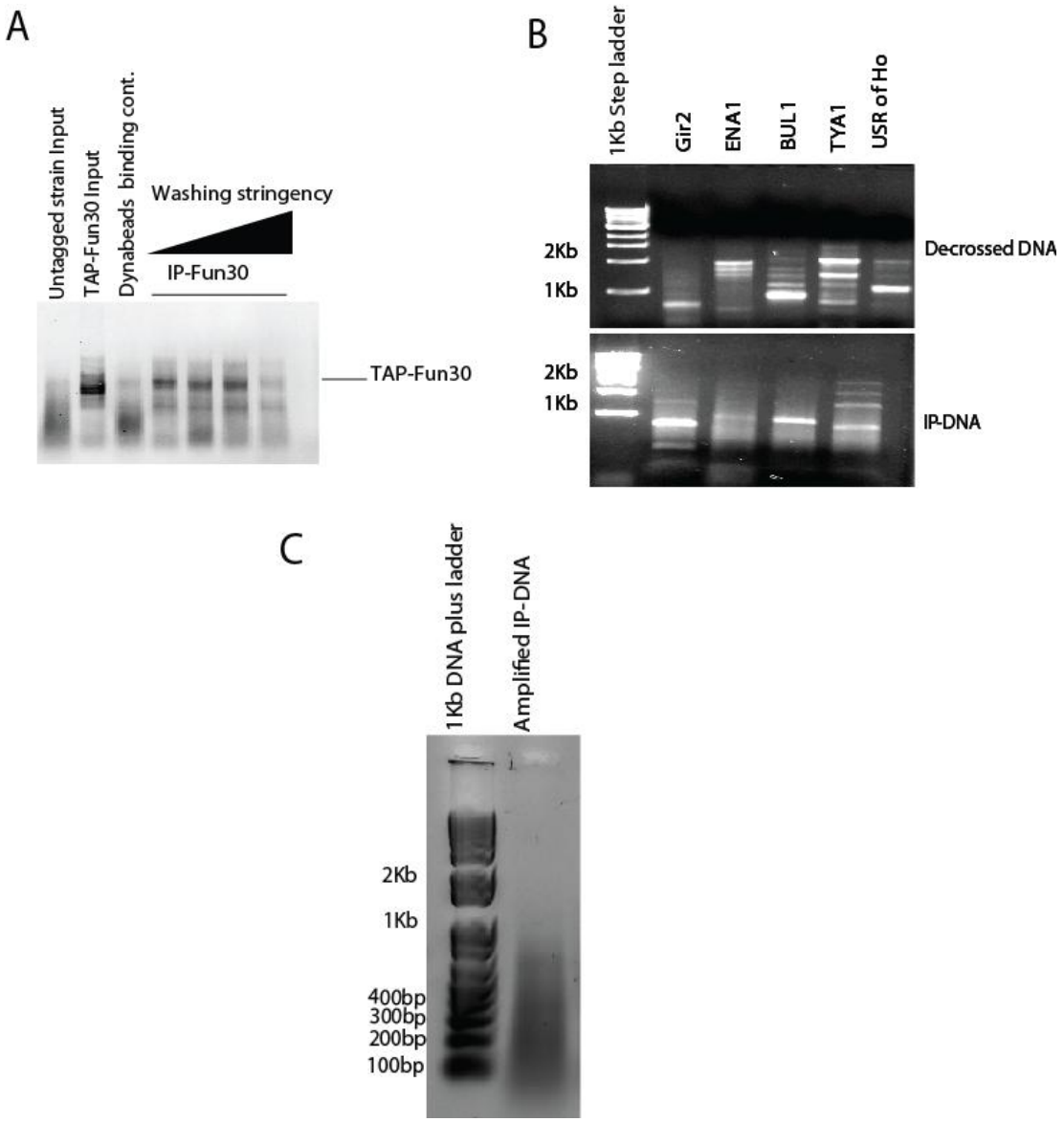

Figure 5.2.1. Optimization of Fun30 ChIP-chip conditions. (A). Western blot analysis using anti-TAP antibody to confirm that IP reaction was specific to pull down Fun30. With increased washing stringency Fun30 tend to dissociate (lane 6 which correspond to the third washing was adopted in this ChIP protocol) . (B). PCR of the IP-DNA using primers constructed to recognize genes identified from the sequencing analysis. DNA from IP-sample and total genomic DNA(de-crossed DNA) was amplified with primers corresponding for Gir2, Ena1, BUL1, TYA1 and USR of HO locus. Amplification was performed for 30 cycles (1 min at 95 °C, 30 sec at 55 °C, and 1 min at 72 °C). The sequencing showed that Fun30 interacts with Gir2, Ena1 and BUL1 genes. (C). Agarose gel of the 2µl of amplified IP-DNA using the GenomePlex® Whole Genome Amplification (WGA) kit from Sigma.

Table 5.2.1. Genes associated with Fun30 as a result of IP-DNA sequencing

Standard gene name/Alias	Name description	Description	Position
GIR2	Genetically Interacts with Ribosomal genes	Highly-acidic cytoplasmic RWD domain-containing protein of unknown function, sensitive to proteolysis, N-terminal region has high content of acidic amino acid residues, putative IUP (intrinsically unstructured protein)	Chr IV
ENA1/ HOR6, PMR2	Exitus NAtru (latin, "exit sodium")	P-type ATPase sodium pump, involved in Na+ and Li+ efflux to allow salt tolerance	Chr IV
TYA Gag and TYB	transposable_element_gene	Retrotransposon that are transcribed/translated as one unit; polyprotein is processed to make a nucleocapsid-like protein (Gag), reverse transcriptase (RT), protease (PR), and integrase (IN); similar to retroviral genes	Chr III
BUL1/ DAG1, RDS1, SMM2	Binds Ubiquitin Ligase	Ubiquitin-binding component of the Rsp5p E3-ubiquitin ligase complex, functional homolog of Bul2p, disruption causes temperature-sensitive growth,overexpression causes missorting of amino acid permeases	Chr XIII

5.2.2. Absence of Fun30 enrichment near ARS region s in exponentially growing cultures

The genome-wide occupancy of Fun30 was first determined in a haploid *S. cerevisiae* strain during exponential growth in rich media. Labelling and hybridization of amplified DNA was performed using an in house service at Nimblgene. We used Nimblgene arrays consisting of the entire genome tiled at a median of 32 bp intervals. Nimblgene operate a two colour system meaning that control and experimental samples are hybridised to the same array. Scanning at the appropriate wavelengths enables the Cy5 and Cy3 signals to be established separately. A normalized Cy5/Cy3 ratio can then be determined, which provides a measurement of Fun30 occupancy. Two biological repeats were sent for hyperdization and signal swap was not performed.

To visualise the distribution of enrichment with respect to various genomic features it is possible to browse data using a genome browser. In order to establish if any trends observed at individual loci have genome wide significance data for all relevant features in the genome need to be aligned. To do this data was imported into a MySQL database. Within this enrichment values per oligo were converted to enrichment per base pair. This enabled comparisons to be made between data obtained in different formats. Also present within the database are records of all standard features including transcriptional and translational start sites and origins of DNA replication. This database was established with support from the Data Analysis Group in the College of Life Sciences, University of Dundee. Analysis of data within the database is then possible, typically involving the construction of scripts designed to address specific questions. In this case scripts were written in Python by Dr.Triantafyllos Gkikopuolos.

As we had previously observed that a *FUN30* deletion resulted in cell cycle arrest at G1/S phase of cell cycle; we investigated the extent to which Fun30 is present at replication origins. To do this Fun30 enrichment was calculated at each base pair spanning 1500bp on either side of the 337 Autonomously Replicating Sequences (ARS) that were determined using SGD. The average signal could then be plotted. The plot shown in 5.2.2 shows no obvious enrichment within the vicinity of ARS sites. As a control enrichment was also measured at sites randomly selected through the genome. As expected Fun30 also showed no enrichment at these sites. (Fig. 5.2.2).

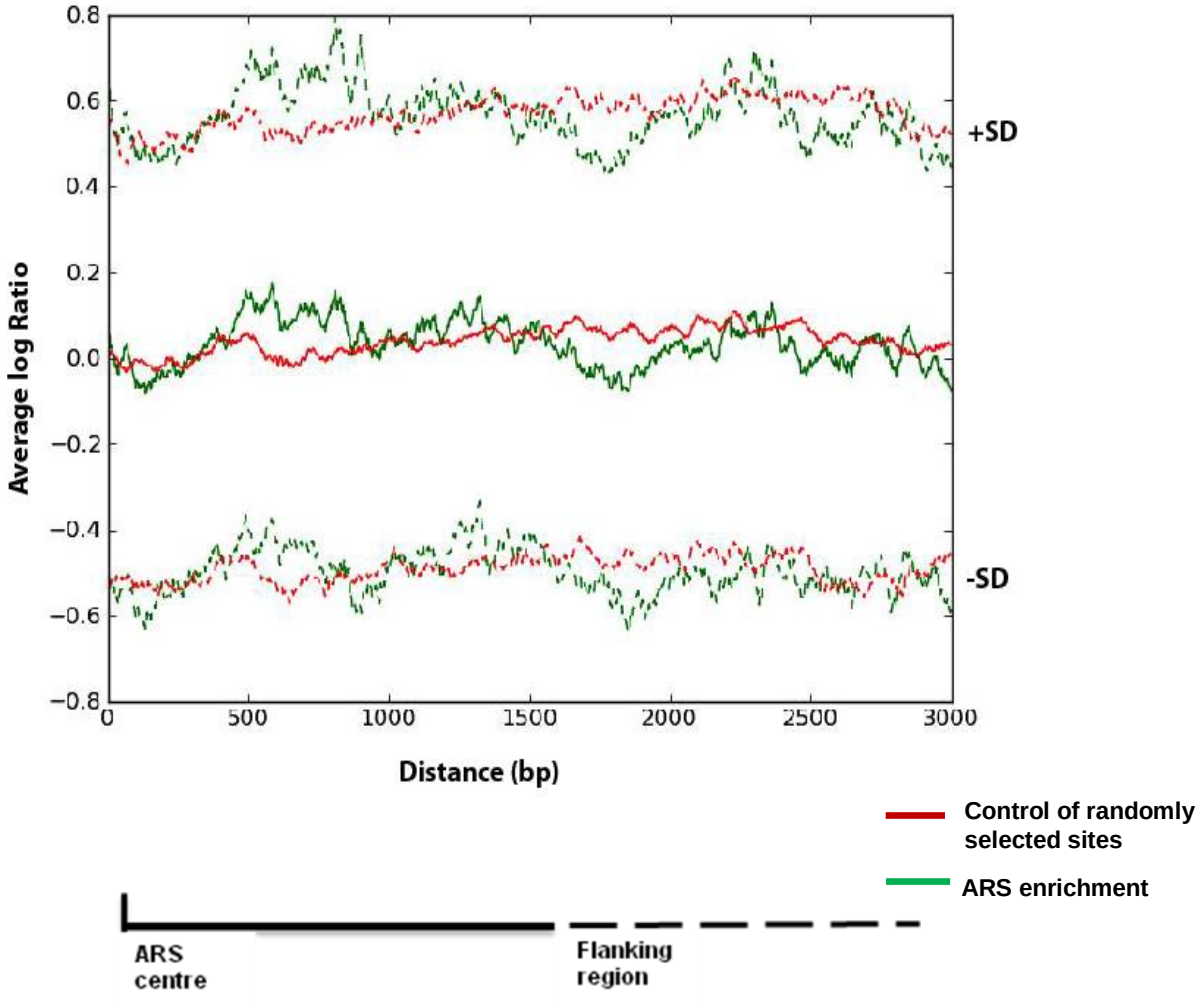

Figure 5.2.2. Genome-Wide Localization pattern of Fun30 near ARS in the asynchronous culture. Fun30 ChIP-chip enrichment localization at ARS± SD plotted as a moving average of the $\log_2$ ratios (y axis) with the distance (x axis). Enrichment of ARS depicting the levels of Fun30 at 1500 bp scale. ARS enrichment detected by the green line was compared to enrichment at randomly selected sites (red line). No difference in the pattern between the –ve SD, +ve SD, and actual experiment lines was observed indicating these values were not significant. Python scripts for this figure were written by Dr.Triantafyllos Gkikopuolos.

5.2.3. Fun30 is located near ARS region in the S-phase of the cell cycle

Since we detected no significant enrichment of Fun30 with respect to replication origins in unsynchronized cultures, we next sought to investigate the effect of cell cycle on the distribution of Fun30. To do this, cells were arrested in G1 using α-factor. Following removal of α-factor, cells were harvested at various stages in the cell cycle and used to prepare Fun30 Chip samples.

No enrichment of Fun30 was observed in the G1/S samples (Fig. 5.2.3. A). On the other hand, a enriched localization spanning a region of approximately 1kb surrounding ARS elements was observed in the S-phase sample (Fig. 5.2.3.B). In the case of the S/G2 time point depletion of Fun30 over this region was observed. These observations would be consistent with transient recruitment of Fun30 to ARS elements during S phase. However, is important to note that although, the degree of enrichment at origins was above the baseline value, it still falls within the standard deviation of the measurement (data not shown) meaning that it may not be statistically significant.

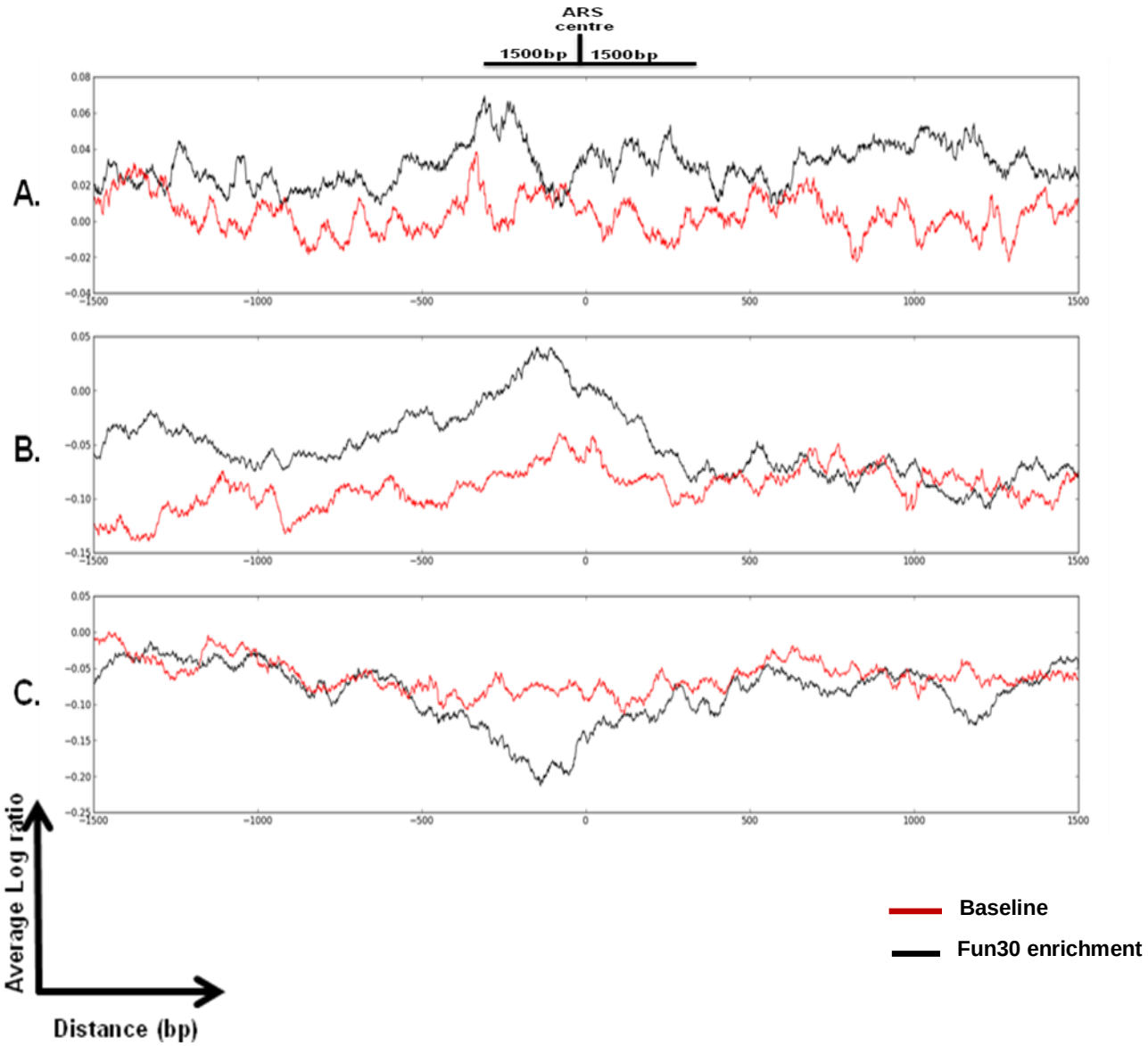

Figure 5.2.3. Fun30 localize at ARS in S-phase of the cell cycle. Fun30 ChIP-chip analysis of the ARS elements at: (A) G1/S phase, (B) S-phase, (C) S/G2 phase of the cell cycle. Enriched localized pattern at ARS are representative of the average Fun30 signal across the ARS. Enrichment values were plotted by the black line in comparison to enrichment at randomly selected sites (red line). Slight increase in the peaks of the black line near ARS center was detected at S and G1 samples. Python scripts for this figure were written by Dr.Triantafyllos Gkikopuolos.

5.2.4. Fun30 localization pattern at ARS changes across the cell cycle

The enrichment values displayed in 5.2.3 represent the mean enrichment at all ARS elements. It is possible that the averaging involved in calculating the mean fails to reveal distinct distributions occurring at subsets of genes. One way of avoiding this is to view the enrichment profiles of individual genes following the grouping ARS elements that show similar distributions by hhierarchical clustering. This indicates that there are differences in the timing and distribution of Fun30 at different clusters of ARS elements.

In general the clustering indicates that regions of strong enrichment or depletion of Fun30 at one time point are preceded or followed by depletion at preceding or following time points (Fig. 5.2.4). These changes in Fun30 occupancy can be classified into several different types of behavior. Some sites are enriched prior to S-phase, these become depleted for Fun30 at later time points. Other sites are enriched for Fun30 during S-phase and interestingly become depleted for Fun30 as cells progress towards G2. Finally, some sites are only enriched at later time points. As a first step towards further characterizing these behaviors, the data were split into three broad groups and the mean enrichment for each time point plotted separately (Fig. 5.2.5). This reveals depletion of Fun30 at later time points for the first two clusters. Although the analysis of this data is preliminary, it is interesting to consider scenarios that might explain these observations. Fun30 might be recruited during the initiation of replication and displaced subsequently. Alternatively Fun30 might be involved in the process of replication which initiates at replication forks and moves away. Finally, Fun30 may be bound on chromatin but removed transiently by passage of a replication fork.

Further analysis of this data will be required. A major problem is the significant noise in the data. This may in part be attributable to differences in the timing of DNA replication. Although it is well known that the entire DNA in eukaryotic cell replicates during the S phase of cell cycle, there is a great variations in the actual point in S phase when a given chromosomal segment replicates. Replication events are known to occur early or late in S phase and it is generally believed that this is determined by the time at which the origins in a segment fire (Bell and Dutta 2002). Even if the conditions in the cell favor the firing of the origins, all origins do not fire at the same time. Thus, our next goal was to determine if Fun30 occupy early or late firing origins (Fig. 5.2.6). We found that Fun30 localizes at ARS of early firing origins with higher enrichment degree during S-phase. However, some enrichment was also detected at the late origins. The enrichment patterns analysis in Fig. 5.2.5-5.2.6 suggested that the Fun30 binding in this stage of the cell cycle was found most frequently, but not exclusively, at origins that might be fire early in S phase.

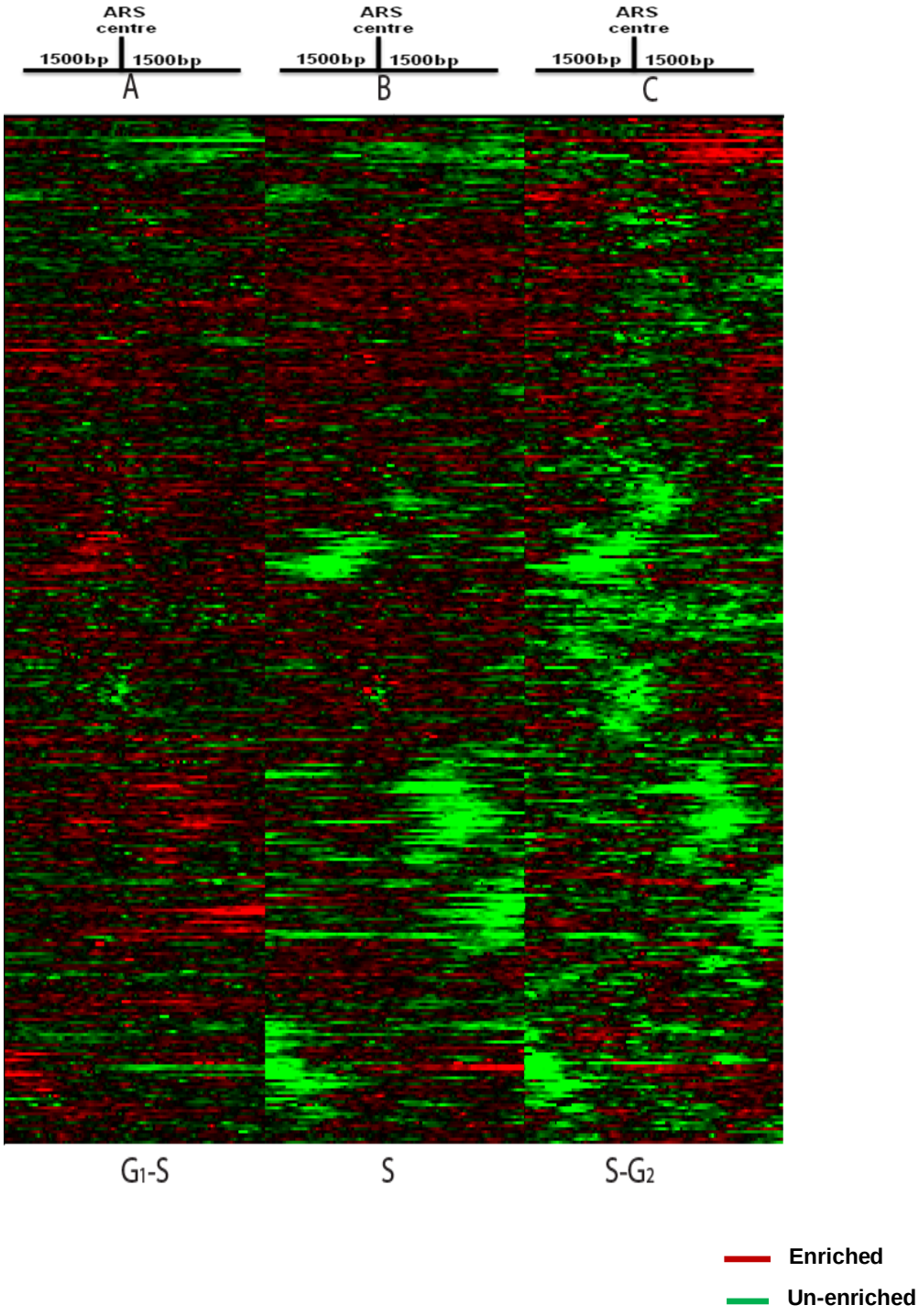

Figure 5.2.4. Fun30 ARS clustering pattern across the cell cycle. Cluster analysis and display of genome-wide Fun30 localization at the ARS in: (A) G1/S phase, (B) S-phase, (C) S/G2 phase of the cell cycle. The data table is represented graphically by coloring each cell on the basis of the measured fluorescence ratio. Cells with log ratios of 0 (ratios of 1.0 − genes unchanged) are colored black, increasingly positive log ratios (enriched) with reds of increasing intensity, and increasingly negative log ratios (un-enriched) with greens of increasing intensity. A representation of the dendrogram is appended to the colored table to indicate the nature of the computed relationship among genes in the table patterns. Python scripts for this figure were written by Dr.Triantafyllos Gkikopuolos.

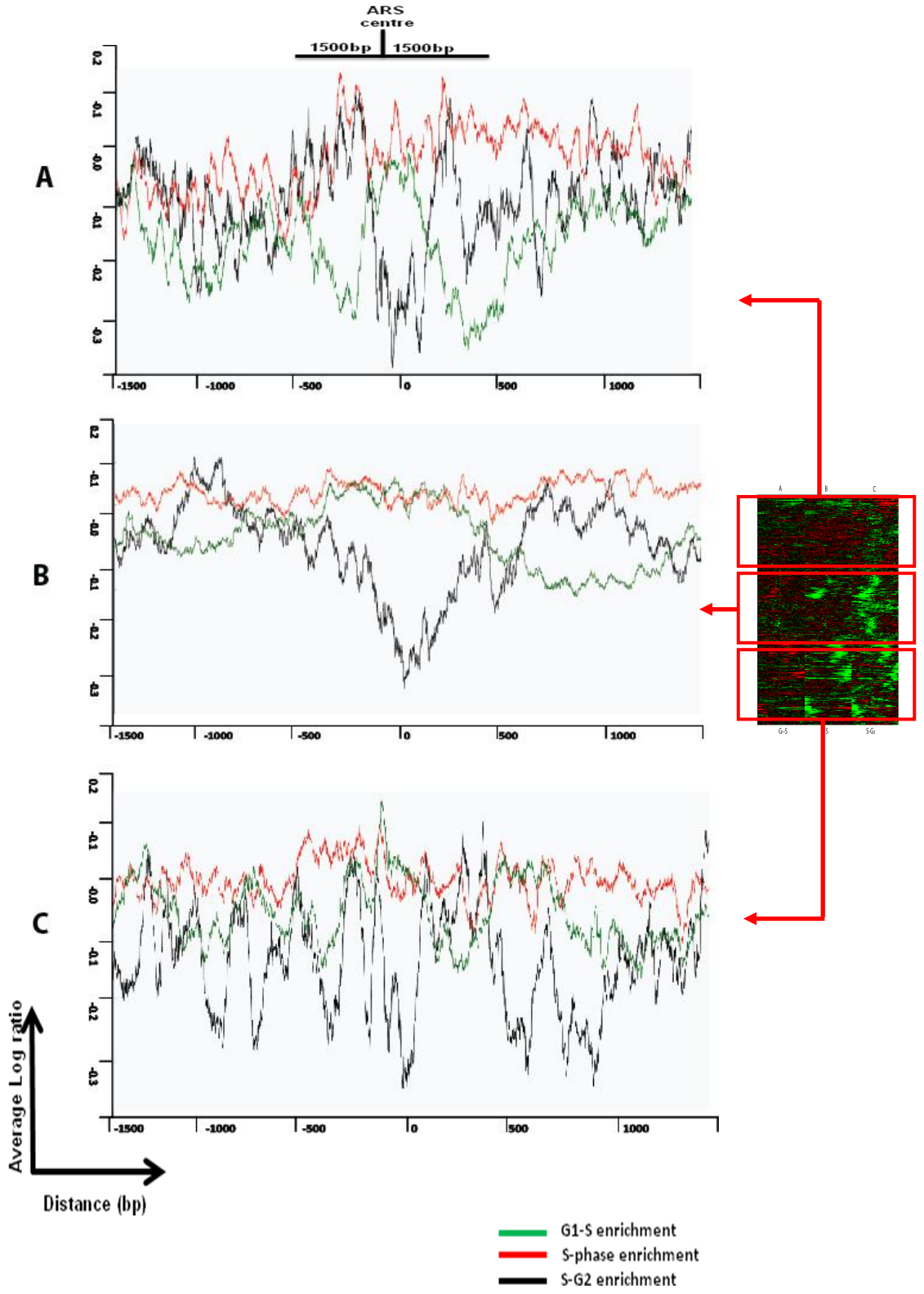

Figure 5.2.5. ARS clustering indicates changes in Fun30 occupancey. Comaprison of ARS clustering obtained in Fig 5.2.4 at the different stages G1/S, S and S/G2 at preceding and following time points. In general ARS from Fig.5.2.4 at the different stages and representing the same location were superimposed to compare the enrichment status at preceding and following time. This analysis indicated that regions of strong enrichment or depletion of Fun30 are preceded or followed by depletion/enrichment. (A). The first 1/3 average of ARS clustering. (B). The second 1/3 of the avarge of ARS clustering. (C). The last 1/3 average of ARS clustering. Python scripts for this figure were written by Dr.Triantafyllos Gkikopuolos.

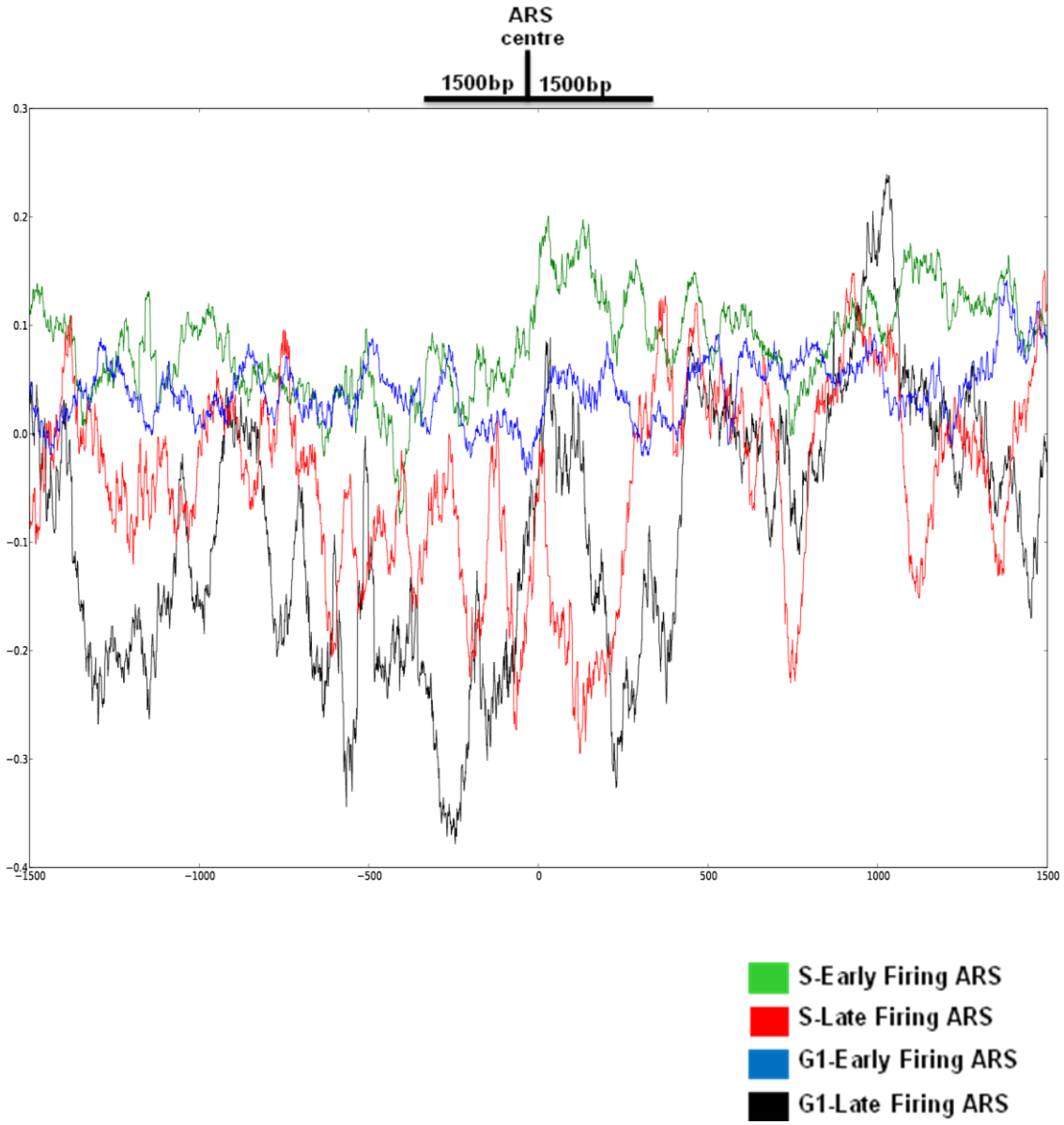

Figure 5.2.6. Early and late firing ARS clustering analysis indicates Fun30 occupancy at early firing origins during S-phase. Comaprison of early and late fring ARS enrichment at G1 and S-phases of the cell cycle. Enrichment values corresponding for late and early firing origion at the G1 and S phases of the cell cycle were plotted. Python scripts for this figure were written by Dr.Triantafyllos Gkikopuolos.

5.2.5. Localization of Fun30 with respect to promoters

Next we examined the pattern of Fun30 localization at promoter regions. We found no significant clustering pattern of Fun30 regarding the occupancy in the promoters in the asynchronous sample (Fig. 5.2.7A). A difference in the distribution of Fun30 was detected in the S-phase IP sample in that clusters of genes with enrichment and depletion could be identified (Fig. 5.2.7B). However, the region of enrichment does not show a strong trend for one region of the promoter over another. This might be anticipated if Fun30 performs a role specific to DNA replication but not relevant to transcription. Moreover, while some regions show enrichment upstream others show enrichment downstream. Clustering will always identify common patterns, but the differences in distribution observed may not be significant.

None the less, a list of genes showing strong enrichment for Fun30 was compiled. When this list of genes was examined, it was found to include genes known to share common roles in the cell such as cell cycle regulation, replication, DNA repair, silencing, ribosomal proteins and stress response (Table 5.2.2). Gene classes of special interest include: Gene silencing (e.g. SAS3, SPP1, and ARD1), genes for cell cycle and replication regulation (e.g. CDC39, GSG1, TOS4, and CAC2), genes involved in RNA synthesis (e.g. RNA15, NAM2, and CDC39), and genes that encode chromatin remodelling and modifying complexes (e.g. SAS3, GIS1, JHD1, ECM32, CAC2, SIP3, ARF3). Two interesting individual hits were the enrichment of SPP1 which is involved in transcription silencing and interacts with Orc2 and the enrichment with RSP5 which is an E3 ubiquitin ligase involved in regulating heat shock response and transcription. Other classes of genes were also notably enriched in Fun30 such as mitochondrial associated partners and ribosomal protein genes (not

shown) and the last is considered a gene class with exceptionally high transcription rates. These observations raise the possibility that Fun30 is associated with transcriptional regulation of a specific subset of genes. However, considerable further work will be required here as there remains a possibility that the association with these genes could be entirely coincidental. For example if the Fun30 is associated with elongating DNA polymerase, association with these genes could occur during the course of DNA replication.

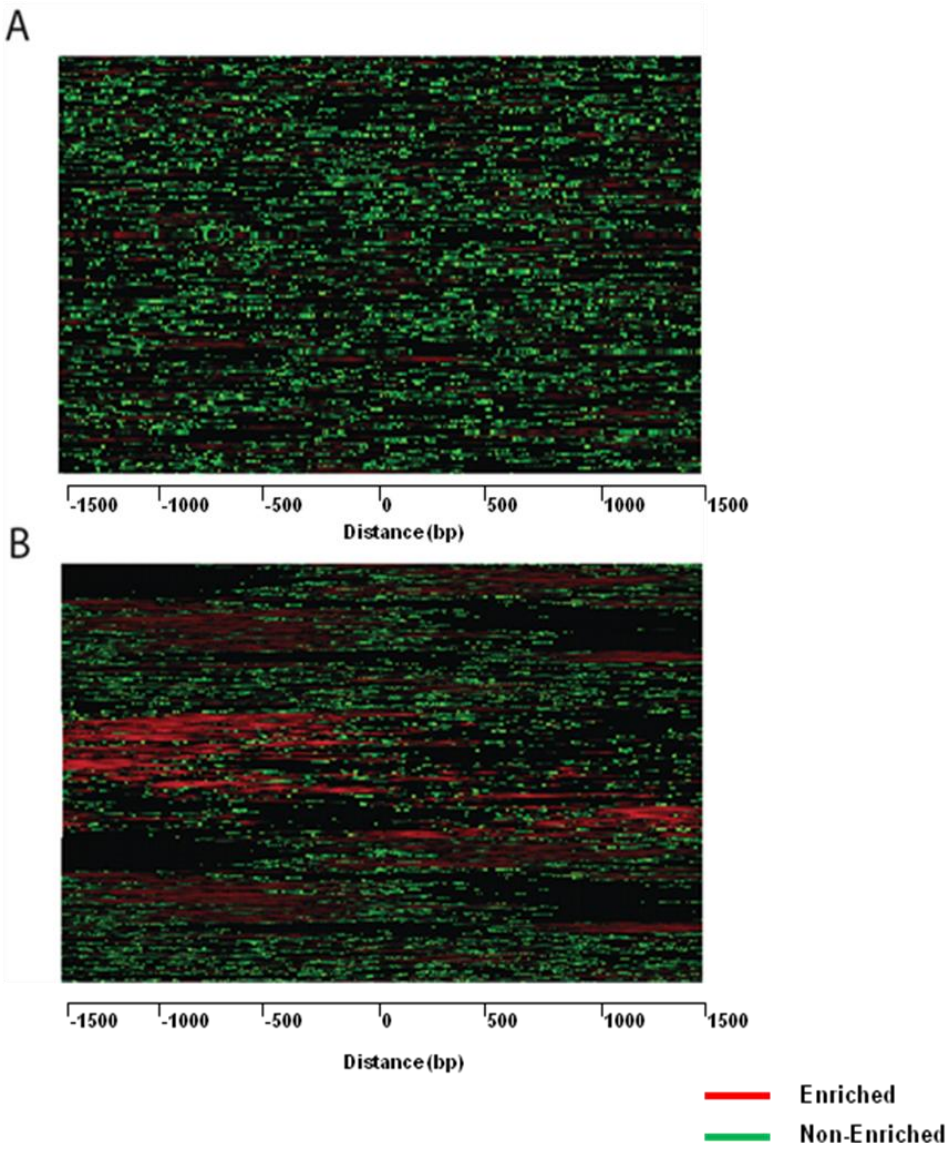

Figure 5.2.7. Fun30 localize near promoters in the S-phase. Clustering pattern of promoters enriched with fun30 at: (A) Asynchronized growth, (B) S-phase IP sample across the genome. Python scripts for this figure were written by Dr.Triantafyllos Gkikopuolos.

Table 5.2.2 Fun30 enriched promoters

Promoters enriched by Fun30	Gene name	Function
SAS3	**Something About Silencing YBL052C**	**Histone acetyltransferase catalytic subunit of NuA3 complex that acetylates histone H3, involved in transcriptional silencing; homolog of the mammalian MOZ proto-oncogene; sas3 gcn5 double mutation confers lethality**
CDC39	**Cell Division Cycle**	**Component of the CCR4-NOT complex, which has multiple roles in regulating mRNA levels including regulation of transcription and destabilizing mRNAs by deadenylation; basal transcription factor**
GIS1	**GIg1-2 Suppressor YDR096W**	**JmjC domain-containing histone demethylase; transcription factor involved in the expression of genes during nutrient limitation; also involved in the negative regulation of DPP1 and PHR1**
GSG1	**General Sporulation Gene YDR108W**	**Subunit of TRAPP (transport protein particle), a multi-subunit complex involved in targeting and/or fusion of ER-to-Golgi transport vesicles with their acceptor compartment; protein has late meiotic role, following DNA replication**
YCF1	**Yeast Cadmium Factor**	**Vacuolar glutathione S-conjugate transporter of the ATP-binding cassette family, has a role in detoxifying metals such as cadmium, mercury, and arsenite; also transports unconjugated bilirubin; similar to human cystic fibrosis protein CFTR**
SOM1	**SOrting Mitochondrial**	**Subunit of the mitochondrial inner membrane peptidase, which is required for maturation of mitochondrial proteins of the intermembrane space; Som1p facilitates cleavage of a subset of substrates; contains twin cysteine-x9-cysteine motifs**
JHD1 /YER051W	**JmjC domain-containing Histone Demethylase**	**JmjC domain family histone demethylase specific for H3-K36, similar to proteins found in human, mouse, drosophila, *X. laevis*, *C. elegans*, and *S. pombe***
RSP5	**Reverses Spt- Phenotype YER125W**	**E3 ubiquitin ligase of the NEDD4 family; involved in regulating many cellular processes, including MVB sorting, heat shock response, transcription, and endocytosis; the human homolog is involved in Liddle syndrome**
GRX4	**GlutaRedoXin YER174C**	**Hydroperoxide and superoxide-radical responsive glutathione-dependent oxidoreductase; monothiol glutaredoxin subfamily member along with Grx3p and Grx5p; protects cells from oxidative damage**
ECM32	**ExtraCellular Mutant YER176W**	**DNA dependent ATPase/DNA helicase belonging to the Dna2p- and Nam7p-like family of helicases that is involved in modulating translation termination; interacts with the translation termination factors, localized to polysomes**

Table 5.2.2. Fun30 enriched promoters (continued)

Promoters enriched by Fun30	Gene name	Function
RNA15	RNA synthesis YGL044C	Cleavage and polyadenylation factor I (CF I) component involved in cleavage and polyadenylation of mRNA 3' ends; interacts with the A-rich polyadenylation signal in complex with Rna14p and Hrp1p
SCW11	Soluble Cell Wall protein YGL028C	Cell wall protein with similarity to glucanases; may play a role in conjugation during mating based on its regulation by Ste12p
CRM1	Chromosome Region Maintenance YGR218W	Major karyopherin, involved in export of proteins, RNAs, and ribosomal subunits from the nucleus; exportin
STE20	STErile YHL007C	Cdc42p-activated signal transducing kinase of the PAK (p21-activated kinase) family; involved in pheromone response, pseudohyphal/invasive growth, vacuole inheritance, down-regulation of sterol uptake; GBB motif binds Ste4p
ARD1	ARrest Defective YHR013C	Subunit of N-terminal acetyltransferase NatA (Nat1p, Ard1p, Nat5p); acetylates many proteins and thus affects telomeric silencing, cell cycle, heat-shock resistance, mating, and sporulation; human Ard1p levels are elevated in cancer cells
NAM2 /MSL1	Nuclear Accommodation of Mitochondria YLR382C	Mitochondrial leucyl-tRNA synthetase, also has a direct role in splicing of several mitochondrial group I introns; indirectly required for mitochondrial genome maintenance
IXR1	Intrastrand cross (X)-link Recognition YKL032C	Protein that binds DNA containing intrastrand cross-links formed by cisplatin, contains two HMG (high mobility group box) domains, which confer the ability to bend cisplatin-modified DNA; mediates aerobic transcriptional repression of COX5b
TOS4	Target Of Sbf YLR183C	Forkhead Associated domain containing protein and putative transcription factor found associated with chromatin; target of SBF transcription factor; expression is periodic and peaks in G1; similar to PLM2
CAC2	Chromatin Assembly Complex YML102W	Component of the chromatin assembly complex (with Rlf2p and Msi1p) that assembles newly synthesized histones onto recently replicated DNA, required for building functional kinetochores, conserved from yeast to humans
SIP3	SNF1-Interacting Protein YNL257C	Protein that activates transcription through interaction with DNA-bound Snf1p, C-terminal region has a putative leucine zipper motif; potential Cdc28p substrate
ARF3	ADP-Ribosylation Factor YOR094W	Glucose-repressible ADP-ribosylation factor, GTPase of the Ras superfamily involved in development of polarity
SPP1	Set1c, Phd finger Protein YPL138C	Subunit of COMPASS (Set1C), a complex which methylates histone H3 on lysine 4 and is required in telomeric transcriptional silencing; interacts with Orc2p; PHD finger domain protein similar to human CGBP, an unmethylated CpG binding protein

5.3. Discussion

Genome-wide localization analysis was undertaken in order to gain insights into the function of Fun30. With no established Fun30 targets to base our studies on, it was important to first establish conditions for efficient immunopurification of Fun30 bound DNA. In our initial shotgun sequencing approach we identified interactions between Fun30 and the the *GIR2* and *BUL1* genes. These interactions were verified by quantitative PCR giving confidence to proceed with a genomewide analysis. Somewhat surprisingly this did not reveal a distinctive pattern of localization with respect to transcriptional start sites. Such patterns have been observed with many proteins known to play a role in the regulation of transcription. This suggested that Fun30 does not play a global role in the regulation of transcription. Fun30 is none the less a relatively abundant protein estimated to present at 6800 copies per cell (Ghaemmaghami et al., 2003). This equates to 1 Fun30 for approximately every 11 nucleosomes. Given that Fun30 is widely conserved amongst eukaryotes this suggests that Fun30 is performing an important function. A striking feature of our ChIP-CHIP data in synchronized cultures is that a far more distinct localization pattern was observed in comparison to data obtained from asynchronous cultures. This could arise if Fun30 acts at specific sites in the genome at specific stages in the cell cycle. In this case the signal from an asynchronous culture might be anticipated to be weaker as a result of averaging.

When the ChIP-CHIP signal was aligned by mapped replication origins, a weak enrichment of Fun30 was observed during S-phase followed by subsequent depletion (Fig. 5.2.3). This type of behavior might be anticipated for factors involved in DNA replication such as the replicative polymerases. They will be recruited to ARS

sequences to initiate replication, but subsequently move away from the origins as replication progresses. Factors localizing to the replication fork would then be anticipated to display peaks of enrichment in regions flanking the origin at subsequent time points. The data in Fig. 5.2.3 do not show peaks flanking the ARS, but instead show depletion in the vicinity of the ARS. This could arise if a factor was required for the initiation of DNA replication, but displaced from DNA once initiation had occurred. This is an attractive concept as Fun30 shows synthetic lethal interactions with several components of the Orc complex including ORC2, ORC3 and ORC5 (Suter et al.,2004; Neves-Costa et al., 2009). However, caution must be taken while interpreting this preliminary ChIP-CHIP data as the enrichment values are low.

There are several potential explanations for this. As discussed in section 5.2.4 heterogeneity in the timing of different origins will mean that the timing of replication is different at different locations in the genome. To try and compensate for this, we grouped related Fun30 enrichment signals into clusters (Fig. 5.2.4). To some extent this supports the idea that the timing or recruitment differs at different locations. However, the patterns of enrichment within these clusters do not provide a precise fit to either an involvement in elongation or initiation of DNA replication. This could be because further analysis is needed to select smaller clusters. It was also interesting to observe that Fun30 localization at ARS was mainly in the S-phase at the early firing origins.

A key stage in the initiation of DNA replication is the binding of ORC (origin recognition complex) to replication origins. In the budding yeast *Saccharomyces*

cerevisiae, ORC recruitment recognizes a specific 11 base pair DNA sequence element within the consensus autonomously replicating sequence (ARS) (Bell and Stillman, 1992). It is known that chromatin is organized with respect to ARS sequences (Eaton et al., 2010). Furthermore, alterations to chromatin organization are known to compromise origin function (Simpson, 1990; Lipford and Bell, 2001). As a result it is perhaps not too surprising that chromatin remodelling enzymes have been implicated in origin function. For example, the SWI/SNF remodelling complex was found to be required for replication initiation in a yeast minichromosome assay (Flanagan and Peterson, 1999). These studies provide indirect evidence that chromatin remodelling may be required to move nucleosomes around the replication origin either to unmask the ORC-binding site, or to configure the nucleosomes around the ORC-binding site to precise positions, allowing ORC to bind and function efficiently.

In order for replication to proceed through chromatin, it might be necessary to pave the way for the replication fork to move without obstacles. In this regard, chromatin remodelling complexes might have an important role during fork movement. Interestingly, two remodelling complexes have been implicated in this process. First, depletion of a human ACF1-ISWI complex (ATP-utilizing chromatin assembly and remodelling factor 1) was shown to impair the replication of heterochromatin in HeLa cells (Collins et al., 2002). This suggests that this complex might facilitate the rate of replication fork progression by remodelling chromatin structure. A second study from the same lab showed that the WSTF (Williams syndrome transcription factor) interacted with PCNA directly to target chromatin remodelling by SNF2h to replication foci (Poot et al., 2004). RNAi depletion of WSTF or SNF2h caused a

compaction of newly replicated chromatin and increased the amount of heterochromatin markers.

Chromatin is reassembled rapidly following DNA replication via reactions that involve histone chaperones such as the CAF1 and ASF1. ATP-dependent remodelling enzymes may also participate in these reactions as enzymes such as ACF and Chd1 have been found to promote the assembly of chromatin in vitro (Lusser et al., 2005). It has been recently shown that Ino80 remodelling enzyme is recruited to replication origins as cells enter S phase (Papamichos-Chronakis and Peterson, 2008). Furthermore, the same study showed that stalling of replication forks in an Ino80 mutant is a lethal event and that much of the replication machinery dissociates from the stalled fork. These data indicated that the chromatin-remodelling activity of Ino80 regulates efficient progression of replication forks and that Ino80 has a crucial role in stabilizing a stalled replisome to ensure proper restart of DNA replication. We found that *in vitro* Fun30 was most active in assays for the removal of histone dimers. This raises the question as to how could this dimer exchange contribute to DNA replication. One possibility is that there is a requirement for a transient incorporation of a histone variant or modification during S-phase. However, we are not aware of candidate modifications on H2A or H2B and we were not able to detect any specificity in the action of the Fun30 enzyme for specific modifications. An alternative is that depletion of histone dimers results in chromatin that is more permissive for replication. This situation could be analogous to events occurring during the elongation of transcription where loss of histone dimers has been observed to be sufficient to allow progression of the polymerase (Kulaeva et al., 2010).

Dissecting which if any of these roles are performed by Fun30 is complicated by the possibility that there will be partial redundancy between Fun30 and other closely related chromatin remodelling enzymes. In budding yeast these include the Ino80 and Swr1 enzymes. Whilst synthetic lethality with components of the Swr1 complex has been detected, this is not possible with the Ino80 complex as it is itself essential. In higher eukaryotes the overlap in subunit composition of complexes containing these enzymes is further evidence for partial redundancy (Fig. 1.2). This may help to explain why the defect following deletion of Fun30 is relatively minor. This emphasizes the importance of relating studies of the defects occurring following deletion of Fun30 to the behavior of the wild type protein.

5.4. Conclusions and perspectives

To address the genome-wide function of Fun30 we performed ChIP-chip analysis. The results obtained from our ChIP-chip analysis show that the genome-wide distribution of Fun30 changes in synchronised cultures. Furthermore some interesting changes in the distribution of Fun30 with respect to ARS sequences were observed. These observations raised the possibility that some of the functions of Fun30 may be linked to DNA replication. In the future it would be interesting to further monitor the effect of Fun30 deletion or degradation on DNA replication directly. This can be achieved by separating replicated and none replicated DNA fragments on density gradients (Raghuraman et al., 2001).

VI. General Closure

The genomes of all eukaryotes are associated with proteins in a compact structure termed chromatin. The structure of chromatin is not uniform, but is manipulated so as to promote or prevent expression of genes. All eukaryotes have complex machineries that are dedicated to altering chromatin structure in different ways. These include enzymes that direct post-translational modification of the histone proteins or DNA, proteins that bind to nucleosomes and alter their properties and ATP-dependent motor proteins that act to non-covalently remodel chromatin structure. ATP-dependent chromatin remodelling enzymes share sequence homology with the superfamily II grouping of helicase related proteins (Eisen et al., 1995 and Flaus et al., 2006). We refer to these proteins as Snf2 family proteins as the yeast Snf2 or Swi2 protein was the first member of this group of proteins to be identified (Flaus et al., 2006). There are 23 broadly conserved subfamilies of Snf2 proteins yet the Function of many of these remains to be determined. A good example is the yeast Fun30 protein. Although this protein is a member of the Snf2 family, the mechanism by which it acts and its biological role are poorly characterised. This project involved an investigation into the Function of the yeast Fun30 protein.

We reported the purification of Fun30 principally as a homodimer with a molecular weight of about 250 KDa. Biochemical characterization of this complex reveals that it has ATPase activity stimulated by both DNA and chromatin. Consistent with this, it also binds to both DNA and chromatin. The Fun30 complex also exhibits activity in ATP-dependent chromatin remodelling assays. Interestingly, its activity in histone dimer exchange is high relative to the

ability to reposition nucleosomes. Fun30 also possesses a weakly conserved CUE motif suggesting that it may interact specifically with ubiquitinylated proteins. However, in vitro Fun30 was found to have no specificity in its interaction with ubiquitinylated histones.

Interestingly, we found that the null mutant of *fun30* resulted in cell cycle arrest at the G1/S transition. Activation of the DNA damage checkpoint in G1/S results in rapid phosphorylation of the yeast checkpoint adaptor proteins Rad 53 and 9 and subsequent binding and recruitment of the checkpoint effectors kinase Rad53, which then transautophosphorylates and becomes active. We observed that null mutant of *fun30* can result in activation of Rad53, which further supporting the notion that *fun30* deletion can result in cell cycle arrest or delay at the early S-phase. Moreover, we have also shown that *fun30* null mutant is resistant to agents that induced DNA damage. These data strongly suggested that *FUN30* plays an important role in DNA repair. *fun30* null mutant cells are sensitive to transcription termination plates which in turn indicate its involvement in this process. Furthermore, we found that fun30 null mutation affected the level of H3 K79 methylation and the histonal ubiquitination status, which indicated its involvement in heterochromatin regulation and ubiquitin interaction. Furthermore, data from ChIP-chip suggested that Fun30 protein is located at replication origins at the S-phase of the cell cycle and it localizes at he early firing origins of replication.

VII. References

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VIII. Appendix

Table 8.1 Mass Spectrometry hits

Protein hit	Mass	Description	Score	Em PAI	Best Match Peptide
gi|6319300	128827	Fun30p (S. Cerevisiae)	413	0.25	K.KESFDAIFK.Q
gi|6320593	93686	Eft2p : Elongation factor 2 (EF-2), also encoded by EFT1; catalyzes ribosomal translocation during protein synthesis; contains diphthamide, the unique posttranslationally modified histidine residue specifically ADP-ribosylated by diphtheria toxin	232	0.14	K.ADLMLYVSK.M
A6ZNY0|A6ZNY0_YEAS7	93686	Translation elongation factor 2(*S.Cerevisiae*)	171	0.09	R.ATYAGFLLADPK.I
gi|296216	13886	H2B	169	1.33	R.KESYAIYVYK.V
Q6AZK7|Q6AZK7_XENTR	13926	Histone 1, H2bk	169	1.33	R.KESYAIYVYK.V
A6ZKU5|A6ZKU5_YEAS7	13981	Histone H2A	146	0.75	R.SAKAGLTFPVGR.V
gi|6323278	116727	Translational elongation factor, stimulates the binding of aminoacyl-tRNA (AA-tRNA) to ribosomes by releasing EF-1 alpha from the ribosomal complex; contains two ABC cassettes; binds and hydrolyses ATP[*S. cerevisiae*]	93	0.04	K.LVEDPQVIAPFLGK.L
gi|4808559	62857	N2,N2-dimethylguonasine tRNA methyltransferase [S. Cerevisiae]	81	0.07	K.YSVAQGPPVDTK.C
gi|14318437	85151	Threonyl-tRNA synthetase, essential cytoplasmic protein [*S. cerevisiae*]	75	0.05	R.IYNTLVDLLR.T
A6ZMP7|A6ZMP7_YEAS7	80850	Chaperonin [*S. cerevisiae*]	72	0.05	K.SVDELTSLTDYVTR.M
gi|6320999	102196	Ubiquitin-specific protease that interacts with Bre5p to co-regulate anterograde and retrograde transport between endoplasmic reticulum and Golgi compartments; inhibitor of gene silencing; cleaves ubiquitin fusions but not polyubiquiti [*S. cerevisiae*]	68	0.04	K.FSEYELLPFK.S
gi|6320956	123038	Kap123: Karyopherin beta, mediates nuclear import of ribosomal proteins prior to assembly into ribosomes and import of histones H3 and H4; localizes to the nuclear pore, nucleus, and cytoplasm; exhibits genetic interactions with RAI1	68	0.03	R.IIEIFSAVFTK.E
A6ZRA8|A6ZRA8_YEAS7	102169	Ubiquitin carboxyl-terminal hydrolase [*S. cerevisiae*]	68	0.04	K.FSEYELLPFK.S
gi|122023	13926	Histone H2B 1.1 (H2B1.1)	66	0.76	K.AMSIMNSFVNDVFER.I

(Protein Sequence matched with SGD http://www.yeastgenome.org)

Table 8.2. List of FUN30 interactions partners

Gene name	Function	Type of interaction with Fun30	References
YPT6	GTPase, Ras-like GTP binding protein involved in the secretory pathway, required for fusion of endosome-derived vesicles with the late Golgi, maturation of the vacuolar carboxypeptidase Y	Synthetic Lethality Negative genetic	Tong et al., 2004 Costanzo et al., 2010
JHD2	JmjC domain family histone demethylase specific for H3-K4, removes methyl groups specifically added by Set1p methyltransferase	Affinity Capture-MS Negative Genetic	Krogan et al., 2006 Collins et al.,2007
ORC2/ORC3/ ORC5	Subunit of the origin recognition complex, which directs DNA replication by binding to replication origins and is also involved in transcriptional silencing; phosphorylated by Cdc28p	Synthetic Lethality Synthetic Growth Defect	Suter et al., 2004 Neves-Costa et al., 2009
SSF1	Constituent of 66S pre-ribosomal particles, required for ribosomal large subunit maturation; functionally redundant with Ssf2p; member of the Brix family	Negative Genetic	Costanzo et al., 2010
RGP1	Subunit of a Golgi membrane exchange factor (Ric1p-Rgp1p) that catalyzes nucleotide exchange on Ypt6p	Negative Genetic	Costanzo et al., 2010
RIC1	Protein involved in retrograde transport to the cis-Golgi network; forms heterodimer with Rgp1p that acts as a GTP exchange factor for Ypt6p; involved in transcription of rRNA and ribosomal protein genes	Synthetic Lethality	Tong et al., 2004
RPN6	Essential, non-ATPase regulatory subunit of the 26S proteasome lid required for the assembly and activity of the 26S proteasome; the human homolog (S9 protein) partially rescues Rpn6p depletion	Affinity Capture-MS	Krogan et al., 2006
RPN5	Essential, non-ATPase regulatory subunit of the 26S proteasome lid, similar to mammalian p55 subunit and to another S. cerevisiae regulatory subunit, Rpn7p	Affinity Capture-MS	Krogan et al., 2006
RPN12	Subunit of the 19S regulatory particle of the 26S proteasome lid; synthetically lethal with RPT1, which is an ATPase component of the 19S regulatory particle; physically interacts with Nob1p and Rpn3p	Affinity Capture-MS	Krogan et al., 2006
CKA1	Alpha catalytic subunit of casein kinase 2, a Ser/Thr protein kinase with roles in cell growth and proliferation; the holoenzyme also contains CKA2, CKB1 and CKB2, the many substrates include transcription factors and all RNA polymerases	Affinity Capture-MS	Krogan NJ et al., 2006

Table 8.2. List of *FUN30* interactions partners (continued)

Gene name	Function	Type of interaction with Fun30	References
PSK2	One of two PAS domain containing S/T protein kinases; regulates sugar flux and translation in response to an unknown metabolite by phosphorylating Ugp1p and Gsy2p (sugar flux) and Caf20p, Tif11p and Sro9p (translation)	Affinity Capture-MS	Ho et al., 2002
MIS1	Mitochondrial C1-tetrahydrofolate synthase, involved in interconversion between different oxidation states of tetrahydrofolate (THF); provides activities of formyl-THF synthetase, methenyl-THF cyclohydrolase, and methylene-THF dehydrogenase	Affinity Capture-MS	Gavin et al., 2002
AAT2	Cytosolic aspartate aminotransferase, involved in nitrogen metabolism; localizes to peroxisomes in oleate-grown cells	Affinity Capture-MS	Krogan et al., 2006
DRS2	Aminophospholipid translocase (flippase) that maintains membrane lipid asymmetry in post-Golgi secretory vessicles; contributes to clathrin-coated vesicle formation and endocytosis; mutations in human homolog ATP8B1 result in liver disease	Affinity Capture-MS	Krogan et al., 2006
LSM1	Lsm (Like Sm) protein; forms heteroheptameric complex (with Lsm2p, Lsm3p, Lsm4p, Lsm5p, Lsm6p, and Lsm7p) involved in degradation of cytoplasmic mRNAs	Two-hybrid	Fromont-Racine et al., 2000
TEL1	Protein kinase primarily involved in telomere length regulation; contributes to cell cycle checkpoint control in response to DNA damage; functionally redundant with Mec1p; homolog of human ataxia telangiectasia (ATM) gene	Affinity Capture-MS	Gavin et al., 2002
CDC28	Catalytic subunit of the main cell cycle cyclin-dependent kinase (CDK); alternately associates with G1 cyclins (CLNs) and G2/M cyclins (CLBs) which direct the CDK to specific substrates	Biochemical Activity	Ubersax et al., 2003
CLB2	B-type cyclin involved in cell cycle progression; activates Cdc28p to promote the transition from G2 to M phase; accumulates during G2 and M, then targeted via a destruction box motif for ubiquitin-mediated degradation by the proteasome	Biochemical Activity	Ubersax et al., 2003
STR2	Cystathionine gamma-synthase, converts cysteine into cystathionine	Negative Genetic	Costanzo et al., 2010
ORM2	Evolutionarily conserved protein with similarity to Orm1p, required for resistance to agents that induce the unfolded protein response; human ortholog is located in the endoplasmic reticulum	Negative Genetic	Costanzo et al., 2010

Table 8.2 List of *FUN30* interactions partners (continued)

Gene name	Function	Type of interaction with Fun30	References
MAD3	Subunit of the spindle-assembly checkpoint complex, which delays anaphase onset in cells with defects in mitotic spindle assembly; pseudosubstrate inhibitor of APC(Cdc20), the anaphase promoting complex involved in securin (Pds1p) turnover	Negative Genetic	Costanzo et al., 2010
SGF11	Integral subunit of SAGA histone acetyltransferase complex, regulates transcription of a subset of SAGA-regulated genes, required for the Ubp8p association with SAGA and for H2B deubiquitylation	Negative Genetic	Costanzo et al., 2010
ARD1	Subunit of the N-terminal acetyltransferase NatA (Nat1p, Ard1p, Nat5p); N-terminally acetylates many proteins, which influences multiple processes such as the cell cycle, heat-shock resistance, mating, sporulation, and telomeric silencing	Negative Genetic	Costanzo et al., 2010
HXT1	Low-affinity glucose transporter of the major facilitator superfamily, expression is induced by Hxk2p in the presence of glucose and repressed by Rgt1p when glucose is limiting	Negative Genetic	Costanzo et al., 2010
PRP4	Splicing factor, component of the U4/U6-U5 snRNP complex	Positive Genetic	Costanzo et al., 2010
OST3	Gamma subunit of the oligosaccharyltransferase complex of the ER lumen, which catalyzes asparagine-linked glycosylation of newly synthesized proteins; Ost3p is important for N-glycosylation of a subset of proteins	Negative Genetic	Costanzo et al., 2010
COG3 COG5 COG6 COG7	Component of the conserved oligomeric Golgi complex (Cog1p through Cog8p), a cytosolic tethering complex that functions in protein trafficking to mediate fusion of transport vesicles to Golgi compartments	Negative Genetic	Costanzo et al., 2010
FIT3	Mannoprotein that is incorporated into the cell wall via a glycosylphosphatidylinositol (GPI) anchor, involved in the retention of siderophore-iron in the cell wall	Negative Genetic	Costanzo et al., 2010
PAP2	Catalytic subunit of TRAMP (Trf4/Pap2p-Mtr4p-Air1p/2p), a nuclear poly (A) polymerase complex involved in RNA quality control; catalyzes polyadenylation of unmodified tRNAs, and snoRNA and rRNA precursors; disputed role as a DNA polymerase	Negative Genetic	Costanzo et al., 2010

Table 8.2 List of *FUN30* interactions partners (continued)

Gene name	Function	Type of interaction with Fun30	References
RAP1	DNA-binding protein involved in either activation or repression of transcription, depending on binding site context; also binds telomere sequences and plays a role in telomeric position effect (silencing) and telomere structure	Negative Genetic	Costanzo et al., 2010
ARP8	Nuclear actin-related protein involved in chromatin remodelling, component of chromatin-remodelling enzyme complexes	Negative Genetic	Hannum et al., 2009
TAF13	TFIID subunit (19 KDa), involved in RNA polymerase II transcription initiation, similar to histone H4 with atypical histone fold motif of Spt3-like transcription factors	Negative Genetic	Collins et al., 2007
CTF18	Subunit of a complex with Ctf8p that shares some subunits with Replication Factor C and is required for sister chromatid cohesion; may have overlapping functions with Rad24p in the DNA damage replication checkpoint	Negative Genetic	Collins et al., 2007
PTA1	Subunit of holo-CPF, a multiprotein complex and functional homolog of mammalian CPSF, required for the cleavage and polyadenylation of mRNA and snoRNA 3-prime ends; involved in pre-tRNA processing; binds to the phosphorylated CTD of RNAPII	Negative Genetic	Collins et al., 2007
SWC3	Component of the SWR1 complex, which exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A; required for formation of nuclear-associated array of smooth endoplasmic reticulum known as karmellae	Synthetic Lethality	Krogan et al., 2003
VPS71	Nucleosome-binding component of the SWR1 complex, which exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A; required for vacuolar protein sorting	Synthetic Lethality	Krogan et al., 2003
ARP6	Actin-related protein that binds nucleosomes; a component of the SWR1 complex, which exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A	Synthetic Lethality	Krogan et al., 2003
HTZ1	Histone variant H2AZ, exchanged for histone H2A in nucleosomes by the SWR1 complex; involved in transcriptional regulation through prevention of the spread of silent heterochromatin	Synthetic Lethality	Krogan et al., 2003
VPS72	Htz1p-binding component of the SWR1 complex, which exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A; required for vacuolar protein sorting	Synthetic Lethality	Krogan et al., 2003

Table 8.2 List of *FUN30* interactions partners (continued)

Gene name	Function	Type of interaction with Fun30	References
RPO21	**RNA polymerase II largest subunit B220, part of central core; phosphorylation of C-terminal heptapeptide repeat domain regulates association with transcription and splicing factors; similar to bacterial beta-prime**	**Negative Genetic**	**Collins et al., 2007**
TMS1	**Vacuolar membrane protein of unknown function that is conserved in mammals; predicted to contain eleven transmembrane helices; interacts with Pdr5p, a protein involved in multidrug resistance**	**Positive Genetic**	**Costanzo et al., 2010**
LRS4	**Protein involved in rDNA silencing; positively charged coiled-coil protein with limited similarity to myosin**	**Negative Genetic**	**Costanzo et al., 2010**
WBP1	**Beta subunit of the oligosaccharyl transferase (OST) glycoprotein complex; required for N-linked glycosylation of proteins in the endoplasmic reticulum**	**Negative Genetic**	**Costanzo et al., 2010**
RAD24	**Checkpoint protein, involved in the activation of the DNA damage and meiotic pachytene checkpoints; subunit of a clamp loader that loads Rad17p-Mec3p-Ddc1p onto DNA; homolog of human and S. pombe Rad17 protein**	**Phenotypic Enhancement**	**Beltrao et al., 2009**
RPC34	**RNA polymerase III subunit C34; interacts with TFIIIB70 and is a key determinant in pol III recruitment by the preinitiation complex**	**Positive Genetic**	**Collins et al., 2007)**
RAD3	**5-prime to 3-prime DNA helicase, involved in nucleotide excision repair and transcription; subunit of RNA polymerase II transcription initiation factor TFIIH; subunit of Nucleotide Excision Repair Factor 3 (NEF3); homolog of human XPD protein**	**Positive Genetic**	**Collins et al., 2007)**
CKS1	**Cyclin-dependent protein kinase regulatory subunit and adaptor; modulates proteolysis of M-phase targets through interactions with the proteasome; role in transcriptional regulation, recruiting proteasomal subunits to target gene promoters**	**Positive Genetic**	**Collins et al., 2007)**
RPC40	**RNA polymerase subunit, common to RNA polymerase I and III**	**Positive Genetic**	**Collins et al., 2007)**

Data in Table 8.2 were complied using the *Saccharomyces* Genome Database (SGD) entry for Fun30 (see http://www.yeastgenome.org/)

Table 8.3 Primers for PCR amplification of DNA fragments

Primers	Description	Sequence
	MMTV nuc A sequence	
54[a]	54 bp upstream extension	TATGTAAATGCTTATGTAAACCA
A0	0 bp downstream extension	ATCAAAACTGTGCCGCAG
A18	18 bp downstream extension	TACATCTAGAAAAAGGAGC
	601.3 sequence	
24W	24 bp upstream extension	ACCCGGACCCTATACGCGG
47W	47 bp upstream extension	GGCGCACTGCAGAAGCTTGGTC
0W	0 bp upstream extension	CTGCAGAAGCTTGGTCCC
W0	0 bp downstream extension	ACAGGATGTATATATCTG

Table 8.4 Primers for PCR amplification of DNA fragments

DNA Template	Experiment	Rational behind choice
GUB	Restriction enzyme accessibility assay	Contains SalI restriction enzyme site.
G5E4	Nucleosomal array binding	Long enough (2.5Kb) to construct nucleosomal array.
0W0	Dimer exchange and binding assay	Generate mononucleosomes with no extra flanking DNA sequence.
0W54	Binding assay	Have 54bp flanking DNA sequence; enable us to study binding preference to linker DNA region.
54A18	Dimer exchange	Generate mononucleosomes with octamer asymmetrically positioned at 18bp to ne end and 54 bp at the other.
54A54	Dimer exchange	Generate mononucleosomes with octamer symmetrically positioned at 54bp to both ends.
54A0	Dimer exchange	Generate mononucleosomes with octamer asymmetrically positioned at 54bp to ne end and 0 bp at the other, so that ability to mobilize octamer from one end to other was studied.
HeLA oligonucleosomes	Dimer exchange, ubiquitin and H2AZ binding preference binding assay	Contains modified histones (e.g. ubiquitylated) and histone variants (e.g. H2AZ).

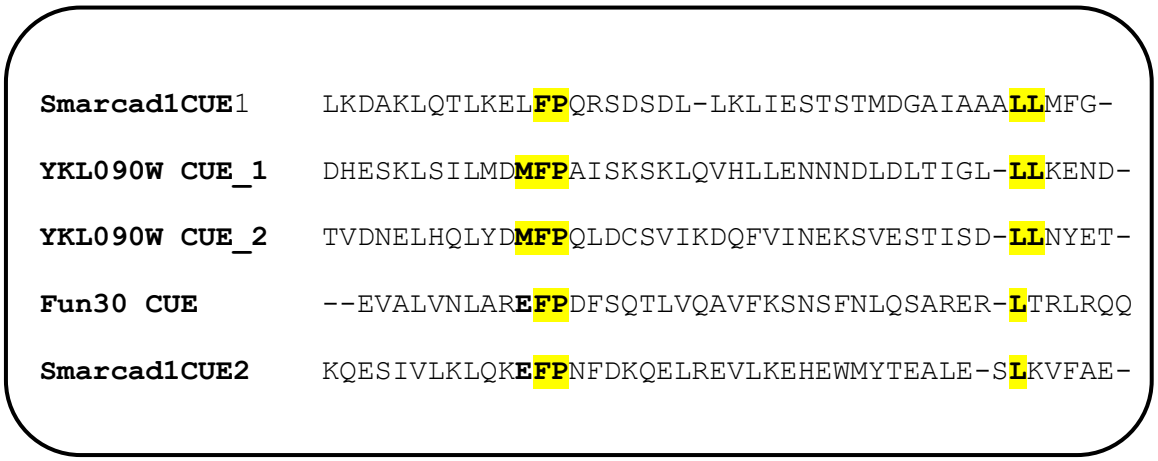

Figure 8.1. Alignment of Fun30 CUE motif with other bone fide Cue domains. The weakly homologus CUE motif of Fun30 was aligned with others known CUE domains using SMART website (http://smart.embl-heidelberg.de/help/smart_about.shtml). A CUE domain FP motif is important for binding to monoubiquitin. The CUE domain invariant proline, the highly conserved di-leucine motif and X-Phe residues that precede the invariant proline are highlighted.

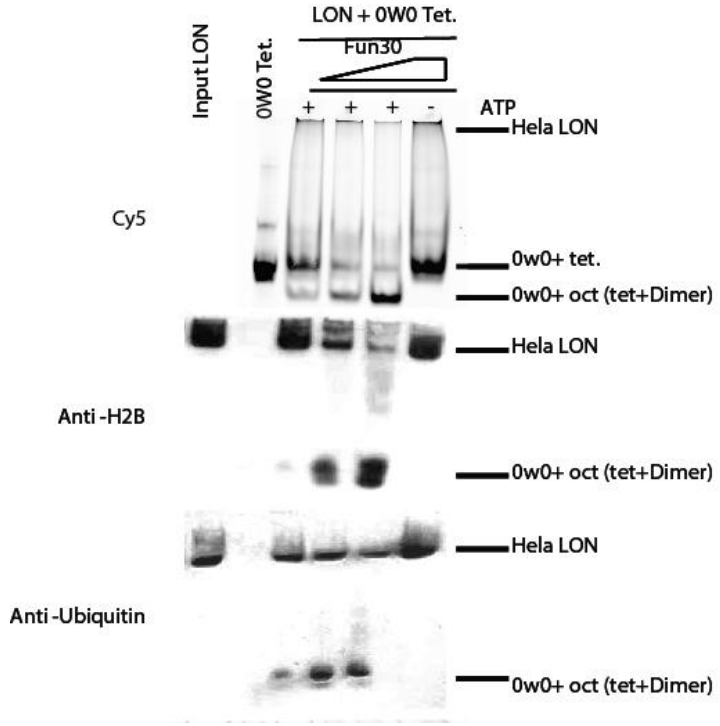

Figure 8.2. Fun30 transfer Hela histones with no specificity towards ubiquitin. A native gel in which ~ 200nM of HeLa mononucleosomes were incubated with increased quantities of Fun30 (~5-320 nM, lanes 3-6) was transferred to a PVDF membrane. The transfer of ubiquitinylated histones and the transfer of total H2B was monitored by Western blotting. No difference in the efficiency with which ubiquitinylated or total H2B is transferred could be detected.

Section 8.1: Labelling of histone mutants containing a unique cysteine

1. Dissolve the lyophilized histone in 900µl 7M guanidinium, 20mM Tris 7.5, 1mM EDTA. Add 100µl 0.5M DTT and incubate at 50°C for one hour.

2. Fill the reaction into a dialysis bag (MWCO 3500) and dialyse against three changes of 10mM NaAc 5.2, 1mM EDTA at 4°C.

3. Remove from dialysis bag and store on ice until the actual labelling reaction is performed.

4. Test labelling:

a) Set up test labelling reactions containing 1nmol of the reduced histone, a variable amount of dye and 50mM HEPES 7.0 (or as desired) in a total volume of 10µl. (I usually try a 0.6 to 1.4 fold molar excess of dye over protein at steps of 0.2) Incubate for 2-3 hours at room temperature.

b) Take 8µl of each test labelling, add 5µl of 7M guanidinium and 13µl of 2x TAU gel loading buffer. Run ~15µl on a TAU gel to determine the minimal dye/protein ratio at which complete labelling occurs.

5. For the large-scale labelling just scale up the successful test reaction. Incubate for 2-3 hours in the dark.

6. Fill into a dialysis bag and dialyse against 1-2 changes of pure H_2O. Aliquot and lyophilise as usual.

Section 8.2; Pi-ATPase sensor assay (Real-time ATPase assay)

The PBP-MDCC (Coumarin based fluorescent phosphate binding protein) is stored at -80C (see freezer list) its now commercially available from Amersham, but is expensive. In future, its probably best to make ourselves. There are detailed notes in papers from the Webb lab, Brune et al (they also recently have a paper on using rhodamine labelled PBP as a phosphate sensor).

- Start reactions by the addition of 1 µl enzyme, with everything else premixed in the cuvette to a final volume of 50 µl, but this can of course be changed.

Everything is in a final buffer of : 50mM NaCl
50mM Tris pH 7.5
1mM MgCl2

- The phosphate sensor is usually 3-5 µM final, and is diluted in 10mM PIPES pH7 to 10x the working concentration. You may need to adjust the final concentration to ensure you can still measure Pi release above the background Pi contamination. You have to test the linear range of the sensor using the phosphate standard, to know that the signal you measure in the real experiment is still linear (at high sensitivity, ~200-450). Remodelling enzyme concentrations tend to be in the range of 0.1-1 nM. Enzymes like RSC have a Km on the order of 10nM for nucleosomes like 36w36. ATP can be either 100uM (probably the highest you can get away with without removing phosphate contamination from the ATP) or 1mM.

Reaction with 1 mM ATP:

5 µl 10X buffer

5 µl 10 mM ATP (Pi-free, see below)

5 µl diluted PBP-MDCC

x µl Substrate (DNA/nucleosomes/octamers etc)

y µl Remodelling enzyme

Make up to 50 µl with H_2O

Fluorimeter settings

Ex 430nm

Em 465nm

Slit-width 5nm

PMT high

Measure every 0.5-1s

Stop time 500s

At room temperature

- To remove free phosphate contamination from ATP (phosphate mop):

To make 200 µl of 10mM ATP

40 µl 50 mM ATP

20 µl 10X standard reaction buffer

20 µl 1M Tris pH 8

120 µl H_2O

- Add 0.8 µl 35U/ µl PNP and 2 µl 30 mM 7-MG (both in the -80 with the PBP), incubate at 30°C for 30 min. Lower [ATP] need less Tris buffering. Dilute at no less than 10 fold into the cuvette or else the mop will strongly interfere with your signal by sucking up Pi released during the ATPase reaction.

IX. PhD Candidate Resume

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Publication(s):

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