Structural Investigation of the Chromatin Remodeller Fun30

Contents

1. Abstract

Manipulation of chromatin structure is closely linked to the process of gene regulation. The core component of chromatin, the nucleosome, consists of approximately 147 base pairs of DNA wrapped around a protein core consisting of two histone H2A-H2B dimers and a histone H3-H4 tetramer. Chromatin structure can be altered through the action of a diverse assortment of proteins and protein complexes including ATP-dependent remodelling enzymes related to the yeast Snf2 protein. A major factor limiting insight into ATP-dependent chromatin remodelling enzymes is a lack of structural understanding. The *Saccharomyces cerevisiae* Snf2 family members Fun30 (Function Unknown Now 30) and Chd1 function as homodimers and monomers respectively and represent tractable systems for structural investigation in comparison to multi-subunit complexes. Here systems are described for expression of Fun30 and Chd1 protein fragments from *Saccharomyces cerevisiae* and the thermophile *Chaetomium Thermophilium*. Conditions were established to express 6 fragments of these proteins in mg quantities. The ability of these fragments to form stable complexes with nucleosomes was also assessed. The formation of stable complexes between Fun30 and nucleosomes indicated the potential for further structural characterisation especially using cryo electron microscopy.

2. Introduction

2.1 Chromatin Structure

In eukaryotic organisms approximately 2 metres of nuclear DNA is packaged within the nucleus in the form of a protein-DNA complex known as chromatin. The interaction between DNA and histone proteins in the form of the nucleosome, the fundamental unit of chromatin, allows for this large amount of DNA to be compacted within the nucleus. The nucleosome consists of 147 base pairs of nuclear DNA wrapped in 1.7 turns around the nucleosome core particle composed of an octamer of histone proteins; two H2A/H2B dimers and one H3/H4 dimer (Kornberg, 1974, 1977). Nucleosomes are joined by stretches of linker DNA forming a 'beads on a string' like structure as observed by electron microscopy (Olins and Olins, 1974). The four histone proteins interact through the histone fold, an evolutionarily conserved dimerization interface composed of a 3-4 turn α-helix, a 7-8 amino acid loop, an 8 turn α-helix, a 6 amino acid loop and a final 2-3 turn α-helix. Histone dimerization occurs through an anti-parallel orientation of their long α2 helices. The central H3/H4 tetramer is enclosed between two H2A/H2B dimers to form the histone octamer, a well conserved structure observed in chicken, fly and budding yeast nucleosome crystal structures amongst others (Harp et al., 2000; White et al., 2001) (Clapier et al., 2008).

There are over 120 discrete protein-DNA interaction sites within the nucleosome and interaction between the nucleosome and DNA is described by SHL (superhelix location) numbers which provide a defined location on the nucleosome. SHL0 is located near the sequence pseudodyad at the points of exit and entry points for the nucleosomal DNA. The SHL number increases by 1 for every turn of the major groove where it faces upward with a range of -7 to +7. DNA binding sites within the octamer conform to two types': α1α1 and L1L2 sites formed by α-helices from adjacent histones and L1 L2 loops respectively. The bulk of DNA interaction occurs from salt links between side chains and hydrogen bonding between main chain amides and the DNA backbone. As DNA-histone interaction occurs primarily with the DNA backbone and not DNA bases it is unlikely to be sequence specific. Nucleosomes have been found to assemble on essentially any DNA sequence but have shown preferential assembly on specific sequences (Davey et al., 2002). A model utilising motifs seen in budding yeast and chicken nucleosomal DNA was capable of identifying half of the nucleosome positions seen in yeast *in vivo* indicating a preference for nucleosome assembly

on these motifs and suggest that this preferential positioning contributes to the formation of specific chromatin domains like promoter regions (Segal et al., 2006) (Ioshikhes et al., 2006). However, other studies have shown that nucleosomes assembled in vitro have a limited specificity for discrete positions and do not conform to the patterns observed *in vivo*, suggesting a non-intrinsic mechanism of statistical positioning contributed to by transcriptional initiation (Zhang et al., 2009, 2010).

Despite the high degree of packaging contributed by the nucleosome core particle further compaction is necessary to package the genome within the nucleus. The next order of chromatin structure is thought to be a 30nm fibre comprised of nucleosomes; however this is a controversial hypothesis. Histone H1 binding has been shown to promote the formation of a 30nm fibre and H1 has also demonstrated a role as a chromatin architecture protein and a recruitment factor for proteins that access and modify the chromatin fibre (Thoma and Koller, 1977; Thoma et al., 1979) (McBryant et al., 2010). An alternative model suggests that chromatin fibres instead form a disordered interdigitated 'polymer melt' based on cryo-electron microscopy imagery of human mitotic cells (Maeshima et al., 2010).

Eukaryotic chromatin structure is altered via a variety of mechanisms including the activity of chromatin remodelling enzymes, the recruitment of histone chaperones, the inclusion of histone variants, the post-translational modification of histones and nucleosomal DNA. These mechanisms act to modify the accessibility and transcriptional potential of chromatin and as a result influence multiple processes within eukaryotic cells.

2.2 Histone Modifications

A series of enzyme families catalyse the covalent modification of chromatin acetylating, methylating, ubiquitinating, sumolyating, phosphorylating and ADP-ribosylating histone proteins. These modifications contribute to a variety of gene regulatory processes. Chromatin modification enzymes are usually large multi-domain proteins with both catalytic and chromatin binding domains essential for their activity (Cruz et al., 2005; Peterson and Laniel, 2004). Histone modifications are reversible with many enzymes that remove histone modifications acting in opposition to pathways that place the modification initially. For example histone acetyltransferases (HATs) and histone deacetlytransferases (HDACs) often belong to pathways that are antagonistic to one another. Histone modifications may also alter

the structure of chromatin changing the accessibility of certain DNA regions and influencing gene expression (Peterson and Laniel, 2004).

Histone methylation is most prominent on lysine 4, 9 and 27 of histone H3 and lysine 20 of H4, these residues can be mono- di- or tri-methylated (Struhl, 1999). H3 K9, and H4 K20 methylation are associated with repressed regions of chromatin; H3 K9 is also linked to the recruitment of HP1 which facilitates heterochromatin formation. At other sites such as H4 R3 histone methylation is associated with the activation of transcription and acetylation (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001; Rea et al., 2000). Histone phosphorylation is also linked to gene activation and plays roles in the condensation of mitotic chromosomes and DNA damage repair. H3 serine 10 phosphorylation plays a role in increasing transcription in *Drosophila* during heat-shock response and the phosphorylation of serine 10, 28 and threonine 11 of H3 is required for mitotic chromosome condensation (Goto et al., 1999; Hendzel et al., 1997; Hooser et al., 1998; Nowak and Corces, 2000; Preuss and Landsberg, 2003; Thomson et al., 1999). H3 phosphorylation occurs after the activation of DNA-damage signalling pathways indicating a role for the modification of chromatin structure in assisting DNA damage repair initiated by histone phosphorylation (Downs et al., 2004).

Ubiquitination regulates several important cellular processes including transcriptional regulation, histone modification and protein trafficking. Loss of ubiquitin from the K123 of yeast histone H2B has been demonstrated to result in defects in meiosis and mitosis indicating an important role for histone ubiquitination in the yeast cell cycle (Robzyk et al., 2000). There are several known ubiquitin recognizing domains including Ubiquitin interacting domains (UIM), ubiquitin associated motifs (UBA), and the CUE motif (coupling of Ubiquitin to ER degradation). H2B K123 ubiquitination has been linked to methylation of H3 K4 and K79 methylation and gene silencing (Briggs et al., 2002; Sun and Allis, 2002).

Histone acetylation is the best understood histone modification due to extensive studies on the process. HATs and HDACs add and remove acetyl groups from histones respectively. A single histone acetylation modification can be seen to control both the interactions between chromatin and non-histone enzymes as well as alter higher-order chromatin structure. Histone H4 lysine 16 acetylation (H4-K16ac) can prevent chromatin from cross-fibre interactions and disrupt the folding of chromatin into 30nm fibres (Shogren-Knaak et al., 2006). There a series of proposed mechanisms for how histone acetylation contributes to transcriptional regulation.

Bromodomains can recognize acetylated residues and are commonly found within transcription factors as well as HATs and chromatin remodelling enzymes (Dhalluin et al., 1999). These domains can non-specifically recognize acetylation but in some cases like the tandem plant homeodomain human DPF3b this recognition is specific for acetylated histones (Zeng et al., 2010). H3 and H4 acetylation is enriched in actively transcribed regions in the genome suggesting a role for HATs as activators of transcription such as the SAGA and TFIID complexes that have a role in the regulation of many yeast genes (Brinkman et al., 2006; Huisinga and Pugh, 2004; Liu et al., 2005; Pokholok et al., 2005). Histone acetylation also has roles unrelated to transcription including DNA damage where H3 K56 acetylation loss or disruption is linked to an increased sensitivity to DNA damaging agents as well as growth defects (Celic et al., 2006; Chen et al., 2008; Driscoll et al., 2007; Han et al., 2007; Hyland et al., 2005; Maas et al., 2006; et al., 2005; Recht et al., 2006).

Histones undergo a series of other less common modifications including ribosylation, adenylation, biotinylation, and sumoylation which are not covered in this introduction.

2.3 Histone Variants

Histone variants that differ from the major histone species are present in all eukaryotes (Redon et al., 2002). These variant histones are linked to specific functions including gene expression, centromere assembly and DNA strand break repair (Redon et al., 2002; Smith, 2002).

H2A.Z is one of the most notable histone variants. While in mammals H2A.Z is essential for development, yeast H2A.Z mutants are viable but display transcriptional defects (Faast et al., 2001; Santisteban et al., 2000). H2A.Z acts to constrain the localisation of silencing proteins by acting as a boundary element (Meneghini et al., 2003). H2AX is another variant of histone H2A. In metazoans H2AX is phosphorylated in response to DNA damage. In budding yeast, phosphorylation of canonical H2A substitutes for H2AX phosphorylation (Lydall and Whitehall, 2005; Shroff et al., 2004). H3 variants include CENP-A a centromeric specific variant associated with more compact chromatin in human cells and H3.3 a variant enriched in active chromatin regions (Black et al., 2004; McKittrick et al., 2004).

2.4 Nucleosome binding proteins

Proteins that have the capacity to bind nucleosomes can alter the structure and function of chromatin. Proteins such as the HMG (high mobility group) proteins can bind nucleosomes and alter their accessibility to regulatory factors changing gene expression patterns within these regions as well as modifying chromatin packaging (Bustin et al., 2005; Catez et al., 2002, 2004). The HMGN class of HMG proteins are the only members of the group able to bind nucleosomes but can do so tightly and interact with the H3 tail and H2B functioning to de-compact chromatin (Bustin et al., 2005) (Trieschmann and Martin, 1998). Histone H1 linker proteins have been demonstrated to perform the opposite function binding chromatin to restrict access to nucleosomes. H1 also has roles in the formation of chromatosomes and chromatin fibres and has an effect on gene expression *in vivo* (Alami et al., 2003; Bharath et al., 2003; Carruthers et al., 1998; Hizume et al., 2005).

2.5 Histone Chaperones

Histone chaperones function to transport histones to various locations within the cell primarily to and from the nucleus. Histone chaperones can be subdivided into 5 main groups based on their structural features: β-propeller chaperones, β-barrel and half barrel chaperones, α/β-earmuff chaperones, β-sandwich chaperones and irregular chaperones that don't conform to any of the other 4 groups (Das et al., 2010). Histone chaperones impede non-specific interaction with histones while controlling the integration of histone proteins with chromatin and modulating the overall histone supply of a cell (Loyola and Almouzni, 2004). The FACT complex can remove histone dimers during transcription and promote RNA Pol II transcription (Belotserkovskaya et al., 2003; Orphanides et al., 1998). Histone chaperones Asfp1 and CAF-1(chromatin assembly factor 1) have been linked to gene-silencing in yeast, CAF-1 can also be seen to be targeted to DNA replication and repair sites (Moggs et al., 2000; Sharp et al., 2001). The histone chaperone Nap1 promotes transcription via the chromatin remodeler RSC leading to nucleosomal disassembly in the presence of ATP and the absence of an acceptor DNA molecule (Lorch et al., 2006).

2.6 Snf2-Related Chromatin remodelling enzymes

Chromatin structure is altered by a series of enzymes that utilise ATP hydrolysis to promote nucleosome rearrangements. The alterations to nucleosome structure include nucleosome repositioning, histone eviction and histone dimer exchange. Yeast Snf2 was the first of these enzymes to be identified and subsequently it has become apparent that all eukaryotic genomes include multiple enzymes related to Snf2. These proteins have been classified into 24 subfamilies based upon the sequence similarity of their ATPase subunits. Subsets of these subfamilies have more closely related ATPase subunits and can be grouped together. These groupings tend to share similar properties. For example, the Snf2 grouping which includes the Snf2 subfamily as well as the Chd1 and Isw1 subfamilies as members of this group share a similar capacity for nucleosomal repositioning (Flaus et al., 2006). Of these 24 subfamilies only the Ino80, Etl1 and CHD1 subfamilies will be covered in this introduction.

2.6.1 The Ino80 Subfamily

2.6.1.1 Ino80

The yeast Ino80 protein was found to associate with 12 polypeptides in a complex referred to as the INO80 complex (INOsitol requiring 80 complex) (Shen et al., 2000). Unusually, this complex has two AAA+ superfamily of helicase proteins, the Rvb1 and Rvb2 subunits, in addition to the Snf2 related Ino80 ATPase. The Rvb1 and Rvb2 subunits are both essential for viability in yeast and other eukaryotes (Shen et al., 2000). The bacterial homologs of the Rvb1 and Rvb2 proteins act as strand separating helicases consistent with this, the yeast INO80 complex also has 3' to 5' DNA helicase activity (Shen et al., 2000). The functional significance of this is not yet known. Biochemically Ino80 is active in nucleosome repositioning (Udugama, 2010) . INO80 complex has also been shown to be capable of acting to remove H2A.Z-H2B dimers from variant nucleosomes (Alatwi and Downs, 2015; Papamichos-Chronakis et al., 2011). A change in the distribution of H2A.Z containing nucleosomes is observed in *ino80* mutant cells (Papamichos-Chronakis et al., 2011). Consistent with the potential role of H2A.Z as a boundary restricting the spread of heterochromatin, Ino80 has been shown to restrict the spreading of euchromatin into silent chromatin (Xue et al., 2015) .

Ino80 mutants demonstrate a hypersensitivity to DNA-damaging agents and may contribute directly to double-strand break repair (Shen et al., 2000). In addition, subunits of the Ino80

complex show genetic interactions with the RAD52 repair pathway and may contribute by evicting nucleosomes at the site of a break (Morrison et al., 2004; Tsukuda et al., 2005) . The recruitment of Ino80 to sites of double stranded breaks is dependent on H2A phosphorylation and acts to promote strand resection (van Attikum et al., 2004). The complex has also been observed to promote release of RNA polymerase from chromatin (Lafon et al., 2015). When Ino80 is artificially recruited to exogenous sites it acts to increase their mobility within the nucleus (Seeber et al., 2013). At present it is not clear how many of these different functions relate to functions of Ino80 at the level of the nucleosome. Insight into this key aspect of INO80 function can potentially be derived from structural and mechanistic studies. This is an area where there have been several recent advances. For example, the arp8 module of Ino80 has been shown to interact with histone H3 (Saravanan et al., 2012) in low resolution models for the intact complex. The Rvb1/2 complex in isolation is shown to form a do-decamer (Silva-Martin et al., 2016) and the complex bound to nucleosomes (Tosi et al., 2013) suggest that the rbv1/2 and Arp8 proteins are involved in nucleosome binding. However, the Arp5/Ies6 module has been shown to be more important for chromatin remodelling activity (Watanabe et al., 2015). The strong evidence linking the INO80 complex to functions at the level of the nucleosome raise the possibility that many of the observed phenotypes may be consequences of a role for Ino80 in regulating the distribution of the histone variant H2A.Z (Papamichos-Chronakis et al., 2011) .

It has been demonstrated that the loss of Ino80 and Swr1 does not affect checkpoint competency whereas a loss of histone acetylation mediated by NuA4 results in G1 persistence suggesting the repair of double strand breaks and their subsequent remodelling are separate events that are both triggered by histone modification (Javaheri and Wysocki, 2006). However more recent findings indicate that ino80 does have a role in the regulation of checkpoint activity (Lee et al., 2015).

Ino80 relocates nucleosomes to central positions on DNA fragments. One reasoning for this activity is the production of single stranded regions during DNA repair. INO80 has also been shown to be required for the S and G2 phase-specific binding of Mps3, a SUN domain protein that acts as a binding site for persistent DNA double strand breaks at the nuclear periphery (Horigome et al., 2014).

2.6.1.2 SWR1

While Swr1 (Swi2/Snf2 related complex) and Ino80 share Arp4 and the Rvb1 and 2 subunits they are also comprised of a number of subunits unique to each protein complex.

Swr1 is a Swi2/Snf2 related chromatin remodeler serving as the main catalytic component of a multi-subunit complex capable of replacing histone H2A dimers with H2A.Z. Genome wide studies have shown a 40% overlap between genes regulated by Swr1 and those regulated by the histone variant Htz1 (the yeast homolog of H2A.Z) indicating a common function of transcriptional regulation shared by H2A.Z and Swr1. Deletion of SWR1 has shown a change in the distribution of Htz1 as well as hypersensitivity to UV radiation, caffeine and methyl methanesulfonate (Kobor et al., 2004; Mizuguchi et al., 2004; Tong et al., 2003; Wu et al., 2005). Swr1 may also have some histone acetylation recognizing capabilities due to the presence of bromodomain containing protein Bdf1 within the complex suggesting a possible connection between acetylated histone residues and the deposition of Htz1 (Pootoolal et al., 2004). The Swr1 complex shared similarities with the chromatin remodelling complex Ino80 including a subunit: Arp4. Both complexes are also recruited to sites of DNA damage which may be due to Arp4's ability to bind H2A P-Ser129 (Downs et al., 2004) . Swr1 interacts with H2A.Z's C-terminal α-helix via the Swc2 subunit of the complex. (Wu et al., 2005) (Hong et al., 2014). This is important for conferring high specificity in the stepwise replacement of nucleosomal H2Az/H2b dimers with H2A.Z/H2B dimers (Luk et al., 2010). This specificity has been reported to be regulated by post-translational modification. When histone H3 is acetylated at K56, the SWR1 complex can also remove H2A.Z dimers from nucleosomes (Watanabe et al., 2013). This may help to explain why H2A.Z is not so highly enriched at promoters in S-phase when K56 is acetylated (Nekrasov et al., 2012) . In addition to interacting with nucleosomes, the Swr1 complex binds to DNA tightly. It has been proposed that this strong DNA binding activity may target the complex to the nucleosome free region at promoters (Ranjan et al., 2013), (Yen et al., 2013). Consistent with this Swr1 is localised to promoters and H2A.Z is enriched at the first nucleosome downstream of the TSS (the +1 nucleosome).

As in the case of the INO80 complex, structural details of the Swr1 complex are emerging. The complex has been shown to engage with nucleosomes and a large conformational change

upon binding has been observed (Nguyen et al., 2013). SWR1 has also been shown to play a role in DNA double strand break relocalisation in G1 and S-phase cells where Htz1 incorporation by SWR1 is necessary for break relocation to repair sites in the nuclear periphery (Horigome et al., 2014).

2.6.2 The Etl1 Subfamily

2.6.2.1 Etl 1

Etl1 (enhancer trap locus 1) is a non-essential chromatin remodeler with high homology to the Swr-1 grouping (Flaus et al., 2006) . Etl1 has been shown to be expressed in early development and its deletion is associated with developmental defects such as skeletal dysplasia, growth retardation, and impaired fertility (Soininen et al., 1992). The human homolog of Etl1 SMARCAD1 (SWI/SNF-related, Matrix-associated, Actin-dependent Regulator of Chromatin, containing DEAD/H box 1) can be placed in the SNF2 family due to multiple highly conserved sequence regions including two DEAD/H box motifs. SMARCAD1 has also demonstrated a series of regions suitable for the mediation of protein-protein interaction as well as a nuclear localization signal. SMARCAD1 expression is ubiquitous with particularly high transcript levels seen in endocrine tissue (Neuhaus et al., 1992) . SMARCAD1 has been mapped to chromosome 4q22-q23m a region linked to several human diseases including soft tissue leiomyosarcoma, hepatocellular carcinoma, and hemato-logic malignancies (Adra et al., 2000). SMARCAD1 has been linked to the increased acetylation of H2A K5 and K8 by CREB-binding protein in *Drosophila* and the subsequent upregulation and transcriptional activation of several genes (Doiguchi et al., 2016).

2.6.2.2 Fun30

The *Saccharomyces cerevisiae* homolog of Etl1, Fun30 (Function Unknown Now 30) shares significant homology with SWR1 and INO80 (Flaus et al., 2006). Yeast Fun30 deletion mutants have demonstrated temperature sensitive defects but remain viable while also showing improved resistance to ultraviolet radiation (Clark 1992, Barton 1994). Fun30 deletion has also been demonstrated to lead to defects in histone variant genome-wide H2A.Z occupancy, including within and in proximity to centromeres (Durand-Dubief et al., 2012). In addition to its helicase motif containing ATPase domain common to all snf-2 related

chromatin remodellers, Fun30 notably contains a possible CUE motif. CUE motifs are capable of interacting with ubiquitinated residues, but this has not been demonstrated for the CUE domain of Fun30 (Neves-Costa et al., 2009, Awad et al., 2010). It has been found to exist primarily as a homodimer with activity in hydrolysing ATP, binding nucleosomes and remodelling nucleosomes in an ATP-dependent fashion (Awad et al., 2010). Fun30 acts in the process of histone dimer exchange (as expected from a protein with high homology to Swr1 and Ino80) and has a lesser ability to reposition nucleosomes, with superior binding affinity for DNA when compared to the nucleosome core particle (Awad et al., 2010) as well as additional roles in DNA end resection of double strand breaks and gene silencing (Costelloe 2012, Neves-Costa 2009).

Fun30 participates in the regulation of transcriptional repression via the sliding of promoter proximal nucleosomes. The length of the nucleosome-free region at promoter regions in the absence of Fun30 is noticeably altered and Fun30 target genes have been shown to have an enrichment of ubH2B associated with gene repression and a lack of gene activating modifications (Byeon et al., 2013). The presence of ubiquitinated histones in Fun30 target genes is particularly interesting as this may be linked to Fun30's conserved CUE motif, possibly suggesting a mechanism whereby this motif may recognize these ubiquitinated histone residues. Fun30 has also been demonstrated to remodel chromatin in regions where no transcriptional repression is displayed indicating that its remodelling activity is independent of transcription.

One specific set of targets of Fun30 silencing are reporter genes found in transcriptionally repressed regions. This activity has been directly linked to its ATPase activity and is notable, as Fun30 is the only remodeler known to silence genes within telomeres, rDNA loci and the HMR. Fun30 has been show to bind within the HMR (Hidden MAT right) and result in a changed structure at the boundary element of the HMR (Neves-Costa et al., 2009). An ability to maintain silenced regions of chromatin by inhibiting the formation of euchromatin in these regions has also been shown with incorrect incorporation of histone variants, changes in histone modification patterns and misregulation of gene expression occurring within these regions in the absence of Fun30 (Strålfors et al., 2011). Fun30 contributes to resistance to genotoxic agents like MMS primarily by aiding in the processing of ssDNA gaps and possibly double strand breaks as evidenced by the recovery of this resistance in the absence of Fun30 by overexpressing Exo1 (Bi, 2012). However resistance to genotoxic agents like hydroxyurea is not recovered by Exo1 overexpression suggesting an alternative mechanism

by which genotoxic resistance is imparted. One proposed mechanism is a model in which Fun30 stabilises chromatin structure making the DNA more reactive to processes like homologous recombination (Bi et al., 2015).

Fun30 has been shown to contribute to homologous recombination by interacting with double strand break ends and subsequently promoting Exo-1 and Sgs-1 mediated end resection (Eapen et al., 2012 ; Chen et al., 2012). DNA end resection increases 3-fold in the presence of Fun30. In its absence deletion of Exo1 or Sgs1 doesn't lower end resection activity any further indicating a direct role for Fun30 in this process. Studies have also shown that Fun30 promoted end resection is restrained in the presence of the histone variant γ-H2A.X. Fun30 also contributes to ending the DNA damage checkpoint in the presence of an unrepaired double strand break (Eapen et al., 2012). SMARCAD1 has also been shown to have similar direct 5' to 3' end resection activity (Costelloe et al., 2012).

Fun30 is involved in the regulation of retrotransposon regulation by altering nucleosome occupancy at retrotransposon-flanking long terminal repeat (LTR) elements. A high-level of nucleosome occupancy is maintained at these elements by Fun30 remodellers leading to the use of downstream transcription start sites and the production of truncated RNA transcripts that are unable to participate in reverse transcription or retrotransposition. This nucleosome occupancy is lowered in stressed cells causing a shift in the TSS (Transcription start Site) and increasing the production of full-length transcripts allowing transposon activity in the genome to be precisely regulated (Persson et al., 2016). In the absence of Fun30, retrotransposon flanking LTR elements and subtelomeres have been shown to lose their position at the nuclear periphery causing a shift in their localisation to the nuclear interior indicating a role for Fun30 in mediating the association between the nuclear envelope and specific chromatin domains (Steglich et al., 2015).

2.6.4 The CHD1 Subfamily

2.6.4.1 CHD1

CHD1 is a member of the CHD (chromodomain-helicase-DNA binding) protein subfamily of ATP-dependent chromatin remodelers. The chromodomains of the human homolog CHD1 bind the bi- and tri- methylated residue of the H3 tail at Lysine 4 however the yeast homolog does not share this ability (Flanagan et al., 2005; Kelley et al., 1999; Sims and Reinberg,

2006), the chromodomains however have been shown to regulate access to the ATPase motor (Hauk et al., 2010). CHD1 has been shown to reposition nucleosomes away from the ends of short linker fragments of DNA in a manner dependent on the H4 N-terminal tail (Ferreira et al., 2007; Stockdale et al., 2006). CHD1 has various roles in yeast including responding to transcriptional stress and in transcription elongation and termination (Lorch et al., 1999; Simic et al., 2003; Zhang et al., 2005). CHD1 has also been identified as a component of the SAGA (Spt-Ada-Gcn5 acetyltransferase) and SLIK (SAGA-like) complexes. One of the two chromodomains of CHD1 specifically associates with K4 on histone H3 a mark associated with transcriptional activity suggesting a role for CHD1 and possibly other chromodomain proteins in the recognition of methylated residues (Pray-Grant et al., 2005). CHD1 is active in the transfer of histones from the NAP1 chaperone to DNA by a processive ATP-dependent mechanism that results in regularly spaced nucleosomes. Further to this the nucleosome repeat length of CHD1 assembled nucleosomes has been found to be shorter than those placed by other remodellers like ACF and ISW1. Genes with longer nucleosomal spacing have higher histone H1 occupancy leading to chromatin folding, the short spacing of CHD1 leads to loss of H1 and chromatin unfolding suggesting a role for CHD1 in the assembly of active chromatin (Lusser et al., 2005) (Ocampo et al., 2016). Structural features that are known for Chd1 include the presence of a Snf2-related ATPase domain, a double chromodomain, and a C-terminal DNA-binding domain with homology to SANT and SLIDE domains previously identified in ISWI remodelling enzymes as determined from crystal structures at 2.0 Å resolution (Ryan et al., 2011). However the full length structure of CHD1 has not been resolved to a high atomic resolution as of yet, with most high quality structural information having been obtained from crystallised truncated domains of CHD1. As a result a tractable full length CHD1 molecule is an attractive target for structural studies.

3. Aims

The primary goal of this project was to investigate the structure of Snf2 family chromatin remodelling enzymes. Members of the Fun30 and Chd1 families were selected as the primary targets as they exist predominantly as homodimers and monomomers rather than as components of large multi-subunit complexes. The first aim was to establish systems for the expression of these enzymes as recombinant proteins. In addition to expressing intact proteins, expression of fragments of both proteins would be investigated as this has the potential to provide structural information on isolated domains. Domains that could be expressed to sufficient homogeneity and purity would be characterised using a range of structural approaches. These include Cryo-EM, Small Angle X-ray Scattering (SAX), Electron Paramagnetic Resonance Spectroscopy (EPR) and Crosslinking Mass Spectrometry.

4. Materials and Methods

4.1 Fun30 Constructs plasmid production

Specific primers for each construct were designed in SerialCloner with BamH1 and EcoR1 restriction sites for the forward primer and Spe1 and Xho1 for the reverse. PCR experiments were set up using the unique forward and reverse primers for each construct, Phusion Hot Start II (Thermo Scientific) and the required template and reagents in 5X Phusion buffer to produce the required Fun30 fragments. The resulting PCR products were purified using a MinElute PCR purification kit (Qiagen). The purified samples were then digested with BamH1 and Spe1 in 2X Tango Buffer for four hours and the digested inserts were ligated to a Pgex6p vector digest (encoding an N-terminal GST-tag and a C-terminal His6) using T4 DNA Ligase. The resulting plasmids were transformed into an XL1 Blue *Escherichia coli* strain (Agilent), colonies produced from this transformation were used to inoculate 10mL LB media (with Ampicillin and Chloramphenicol) cultures. Plasmid preparations were then carried out on these cultures to produce DNA stocks of the Double-tagged Fun30 fragment plasmids (N-terminal GST-tag, C-terminal His6) using a Qiaprep Spin Miniprep kit (Qiagen).

4.2 Expression and Purification of Recombinant His-tagged Fun30 (and Fun30 constructs)

Double-tagged Fun30 (N-terminal GST-tag, C-terminal His6) was in expressed in a Rosetta2 *Escherichia coli* strain (Novagen) at 21°C over 48 hours in Studier autoinduction media (with Ampicillin and Chloramphenicol). Cells were harvested by centrifugation at 5500rpm for 25 minutes and resuspended in lysis buffer (40mM HEPES, 5mM $MgCl_2$, 250mM NaCl, .5mM β-mercaptoethanol). Cells were lysed by freeze/thawing in liquid nitrogen, addition of a 1:100 dilution of a protease inhibitor mix (5% AEBSF, E64, aprotinin, pepstatin) along with DNAse addition (ratio of 1mg per litre culture) and subsequent sonication. The soluble fraction was then extracted by centrifugation at 21,000rpm at 4 °C for 30 min. GST-Fun30-His6 was then purified using His-Pur cobalt resin, washed in lysis buffer, and eluted in lysis buffer containing 250 mM imidazole. The His-Pur elution was then applied to Super Glu-glutathione resin (Pierce) and washed in lysis buffer, before resuspension of the Super Glu-glutathione resin in lysis buffer containing 5 mM DTT, EDTA, EGTA and Precission Protease (ratio of 100µg per litre culture). Fun30-His6 is released from the resin by GST tag

cleavage overnight in this buffer before concentration in 50-kDa molecular mass cut-off centrifugal concentrators and further purification via gel filtration chromatography.

4.3 Octamer, tetramer and dimer assembly and purification

Octamer, tetramer and dimer assemblies were performed following a published protocol (Luger et al., 1999) with minor changes. Firstly lyophilised histones were dissolved in 500 µl of unfolding buffer (7 M guanidinium hydrochloride, 20 mM sodium acetate pH 5.2, 10 mM DTT). Their absorbances were measured at 280nm and the histones were mixed in an equimolar ratio. Samples were dialyzed using a 6000-8000 Da MWCO membrane (SpectraPor 2.1, USA) at 4 °C 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 3 hours stirring per 1 L. Samples were centrifuged in a 4 °C Eppendorf benchtop centrifuge for 10 minutes at 20817 g, before being concentrated to 200-300 µl with a YM-10 Centricon spin concentrator (Millipore, USA). 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, dimers at 16.3 ml, tetramers at 14.2 ml and octamers elute at 13.3 ml. The pooled fractions were concentrated to 20-30 µM for octamer and tetramer assemblies and 80-100 µM for dimer assemblies with a YM-30 Microcon spin concentrator (Millipore. Samples were stored at 4 °C.

4.4 Generation of fluorescently labeled dimers

Dimers were fluorescently labeled by attaching a cy5 mono maleimide dye (GE Healthcare) to a cysteine inserted at H2A. The dimers were prepared as described in Section 4.3 with minor alterations. The samples were heated at 50 °C for 20 minutes after the histones were mixed but before dialysis to further reduce the cysteine 62 residue and ensures their complete unfolding. Unfolding buffer contained 50 mM DTT and the refolding buffer B for dialysis and gel filtration contained no β-ME or DTT and 10 mM sodium acetate pH 5.2. The cy5 dye was dissolved in 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 using 6000-8000 Da MWCO dialysis membrane at 4 °C against 3 *X* 1 L refolding buffer. The volume following dialysis was utilised for the estimation of the dimer concentration.

4.5 Preparative PCR

PCR (polymerase chain reaction) was used to generate large quantities of specific DNA fragments for nucleosome assembly. All of the DNA fragments were amplified from 2 source sequence plasmids; the MMTV nucA (nucleosome A) sequence in a pDONOR201 plasmid (Invitrogen) and the artificial 601.3 positioning sequence in a pGEM-3Z plasmid (Promega, USA) provided by the Widom lab (Anderson et al., 2002). The DNA fragments are defined in the nomenclature as *aBc*. *B* denotes the sequence origin, with the W and the A representing the 601.3 and MMTV nucA sequence, respectively. *a* and *c* represent the upstream and downstream bp extensions on either side of nucleosome core, respectively. 5 ml PCR reactions were assembled on ice with 0.4 ng/µl template plasmid, 1 µM of each primer, 1 *X* NH4 PCR buffer (16 mM ammonium sulphate, 0.01 % Tween-20, 67 mM Tris pH 8.8), 120 µM dNTPs (Bioline, USA), 2 mM magnesium chloride and 20 µl DNA *Taq* polymerase. 5% (v/v) DMSO was added for the amplification of fragments from the 601.3 template. The reaction was transferred into a ThermoFast 96 well low profile plate (ABgene, UK) in 50 µl aliquots and incubated in a Eppendorf mastercycle at 94 °C for 2 minutes, then 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute, with a final incubation at 72°C for 5 minutes.

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

4.6 Purification of PCR products by ion exchange chromatography

Anion exchange chromatography was carried out using a 1.8 ml SOURCE 15Q column (GE Healthcare) on a BioCAD Sprint (Applied Biosystems, USA). A 2 stage 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) was used to separate the DNA fragments from primers and dNTPS. 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 a first gradient of 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 fractions of interest were pooled and ethanol precipitated as described in Section 4.5. The pellet was resuspended in a small volume of ion exchange buffer A and the 260 nm absorbance was measured to determine the concentration, before the sample was stored at -20 °C.

4.7 Chromatin assembly

Chromatin was assembled onto the GUB (Gal USF pBend) template from purified HeLa oligonucleosomes by dilution from high salt (Hassan et al., 2006). Recombinant histone octamers were assembled from *Xenopus laevis* histones expressed in bacteria (Luger et al., 1997).DNA synthesised by PCR from the MMTV nucleosome A was used to reconstitute octamers onto nucleosomes (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 0 M. H2B was fluorescently labelled by attachment of mono maleimide dye (GE Healthcare).

All recombinant histone assemblies were performed by dialysis against increasingly lower salt solutions using a custom microdialysis apparatus and 6000-8000 Da MWCO dialysis membrane at 4 °C. 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 stirring for at least 1.5 hours at each step. The final dialysis performed overnight and the nucleosomes were stored for up to a month at 4°C.

5. Results

5.1 *Saccharomyces cerevisiae* Fun30

5.1.1 Fun30 Constructs

Fun30 is attractive as a model protein to investigate the structure of chromatin remodelling enzymes within the Swr1 grouping of enzymes that are likely to share a function in histone exchange as it acts as a single polypeptide rather than as a component of a multi-subunit complex. With this aim, a series of Fun30 constructs were designed for the purposes of investigating the structure and function of Fun30. Constructs were designed to lack various features of the full length protein in order to investigate how these deletions affect the activity and behaviour of the protein as well as to allow specific structural features of Fun30 to be studied in isolation. Due to the limited structural information available for Fun30 deletions were chosen on the basis of conserved regions between Fun30 and its homologs in other model organisms as well as between Fun30 and other ATPase dependent chromatin remodelers (see Figure 5.1.1). Regions of specific interest include the helicase-like C-terminal ATPase domain and the Cue domain contained within the mostly disordered N-terminal region of Fun30. Also of interest was the possible second N-terminal CUE domain, mammalian Fun30 homologs such as ETL1 and SMARCAD1 are known to contain tandem CUE domains and the presence of a second CUE domain may be significant for optimal Fun30 activity. Four Fun30 mutants were designed to studies these regions as detailed in Table 5.1.

Fun30 Mutant:	**Encoded Amino Acids:**	**Corresponding Region:**
ΔN112	112-1131	Full length Fun30 minus the CUE domain
ΔN259	259-1131	Full length Fun30 minus the tandem CUE domains
ΔN550	550-1131	C-terminal Helicase region
ΔC550	1-550	N-terminal region

Table 5.1. **Fun30 constructs.** This table lists the *S. cerevisiae* Fun30 mutants designed for this project (left column), the amino acids of Fun30 that they encode (middle column) and the regions of Fun30 those amino acids correspond to (right column).

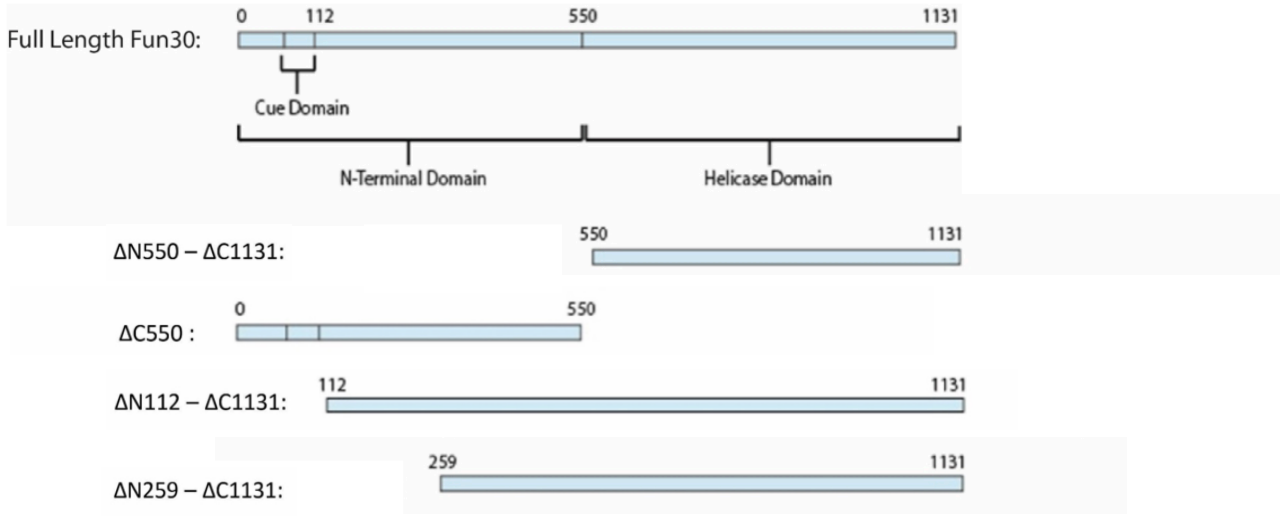

Figure 5.1. **Schematic representation of Fun30 and the Fun30 constructs outlined in Table 5.1.1.** A schematic representation of the known structure of Fun30 illustrating the mostly disordered N-terminal region, the CUE motif located within this region and the Snf2 related ATPase/helicase domain. The four Fun30 constructs designed for this project are also illustrated indicating which regions of the protein are present in each construct.

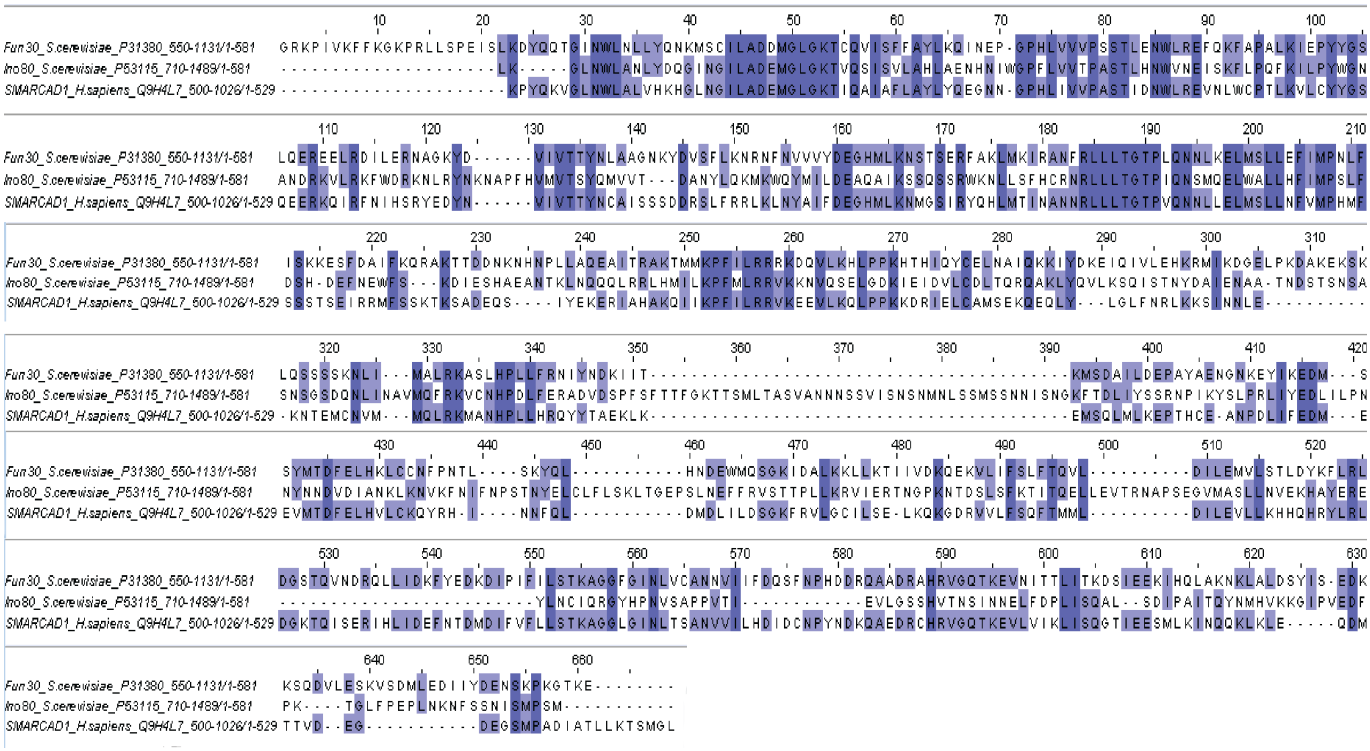

Figure 5.1.1 **Homology between the ATPase regions of Fun30, Ino80 and SMARCAD1.** A multiple sequence alignment of the C-terminal ATPase regions of, from top to bottom, Fun30, Ino80 (a well characterised Swi/Snf chromatin remodeler) and the human homolog of Fun30 SMARCAD1 in JalView. Areas of significant homology are highlighted in the figure.

5.1.2 Fun30 Constructs Plasmid Preparation

Plasmids encoding the four Fun30 constructs were prepared using a Pgex6p vector plasmid. The Pgex6p plasmid was chosen as it encodes an N-terminal GST tag and a C-terminal polyhistidine tag. The double tag purification system using these specific tags has been well characterised for use in the purification of recombinant full length Fun30 as well as other chromatin remodelers and allows these constructs to be purified in an efficient manner with a minimal loss of protein.

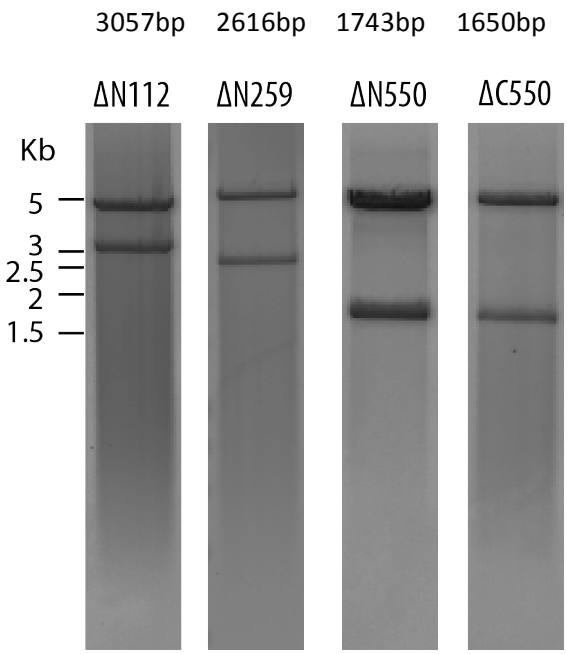

Figure 5.2. **Restriction digest of Fun30 construct plasmids with BamH1 and Spe1.** This figure illustrates the successful integration of the Fun30 constructs into the Pgex6p expression vector. Digestion with BamH1 and Spe1 separates the insert from the Pgex6p plasmid backbone. Pgex6p is ~5Kb in size while the inserts (from right to left) are 3057bp, 2616bp, 1743bp and 1650bp. Correct integration was verified by DNA sequencing of the plasmid.

5.1.3 Expression and Purification of Fun30 and the Fun30 Constructs

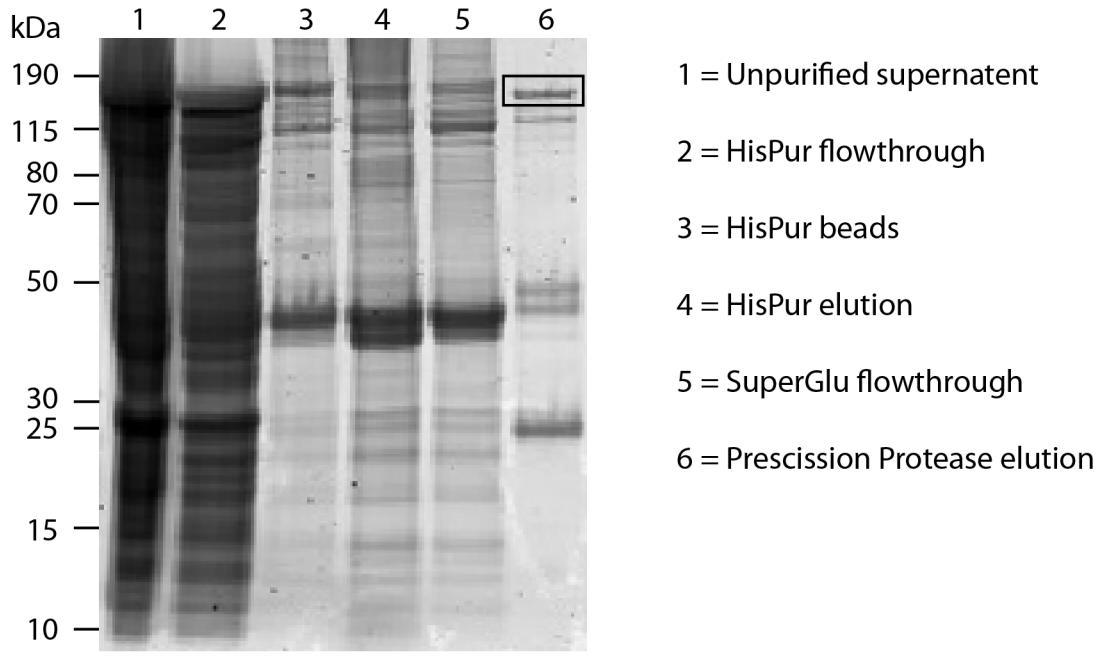

Figure 5.3. **Purification of recombinant full-length Fun30.** Six litres of autoinduction media inoculated with Fun30 was harvested and purified as per the protocol detailed in the methods section. 10µl samples were collected at six steps in the protocol as detailed in the figure legend and run on a 4-12% bis-tris gel. The final band of interest indicating full-length Fun30 after GST tag removal (~130kDa) is highlighted in the figure.

Figure 5.3 details the full purification process for double tagged Fun30 and the stepwise enrichment of the protein after each step of the protocol. While contaminating bands possibly formed of truncated Fun30 can be seen at 48KDa the resulting protein is still of sufficient purity for structural studies. Initial test expressions indicated that all of the Fun30 constructs could be expressed and purified in a similar manner to the full-length protein (Fig. 5.4). Gel filtration chromatography separates the purified protein from other molecules still present in the sample following GST-tag cleavage and the resulting chromatogram can be used to infer structural information about the protein (Fig. 5.5).

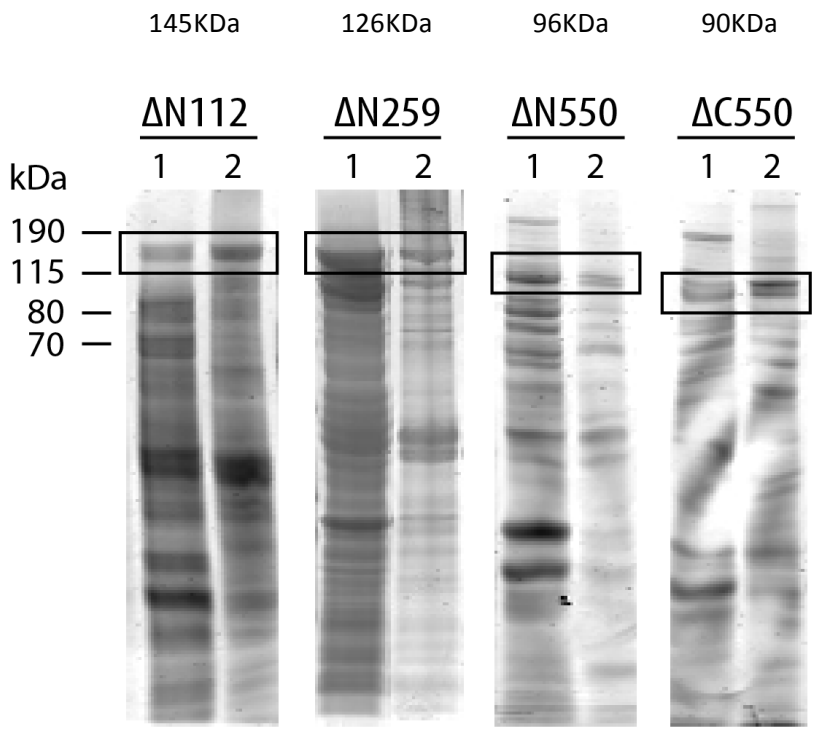

Figure 5.4. **Test Expression of Fun30 constructs.** The four Fun30 constructs were expressed in 150ml autoinduction cultures with ampicillin and chloramphenicol overnight @ 21°C. The cultures were centrifuged at 21,000rpm following lysis and 10µl of the resulting supernatant was run on a 4-12% bis-tris gel as sample 1. 10µl of the HisPur elution following binding of the protein to HisPur cobalt resin was run as sample 2. The bands of interest are highlighted for each construct. The Fun30 constructs from left to right are ~145kDa, ~126kDa, ~96kDa and ~90kDa in size.

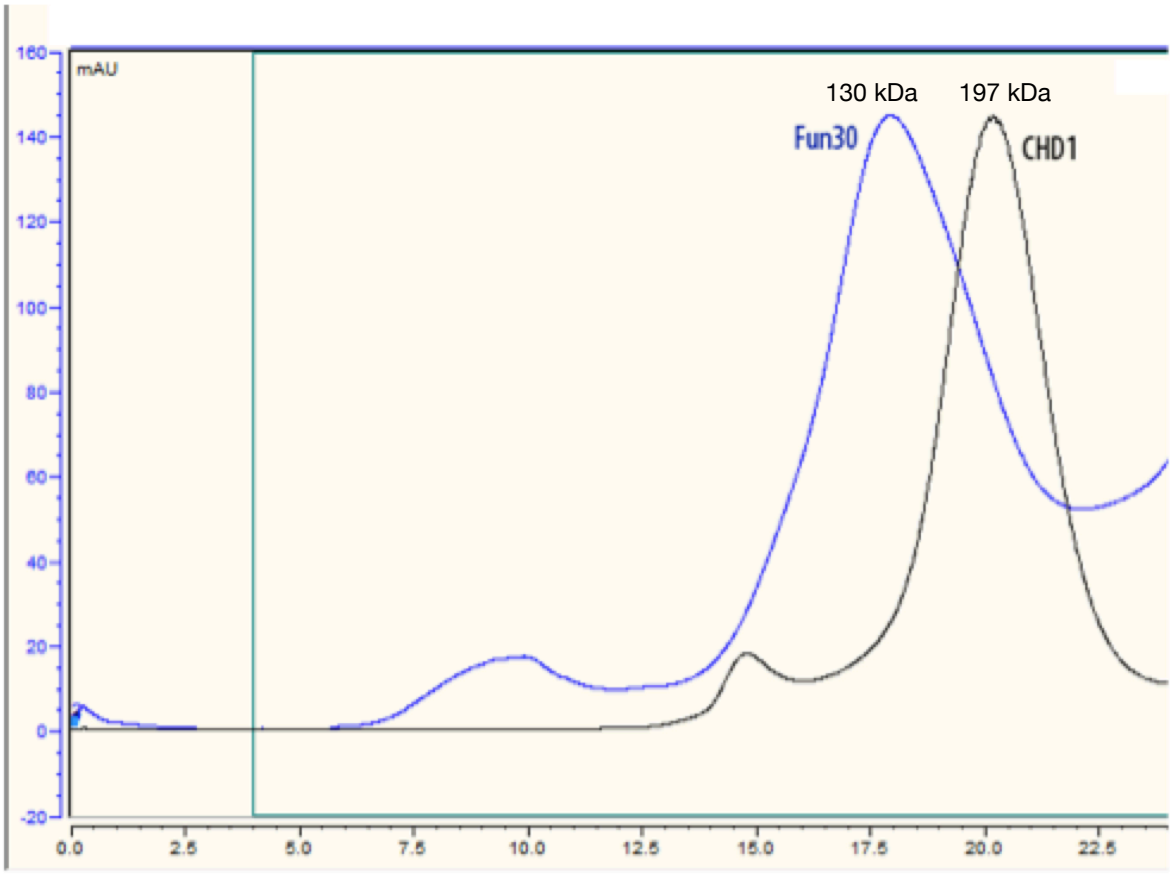

Figure 5.5. **Fun30 is purified as a homodimer.** Fun30 is purified using gel filtration chromatography as a final step in the purification process. In the above figure the chromatogram of purified Fun30 is compared to that of another chromatin remodeller CHD1 with a known structure and size. The recombinant Chd1 sample is ~197 kDa in size but elutes after the smaller (~130kDa) Fun30 sample.

5.1.4 Cryo-Electron Microscopy of the Fun30-Nucleosome complex

Single particle Cryo-EM offers the most valuable approach for the study of single protein complexes. With this method data comprised of 2D projection images of a protein complex in a series of differing orientations can be combined to assemble a 3D model of the overall complex structure. A pilot experiment was performed with a 2mg/µl solution of the purified Fun30-Nucleosome complex (at a ratio of 1µM Fun30 to 30nM nucleosome prior to purification as seen in Figure 5.6) The Nucleosome binding gradient in Figure 5.6 shows two gel shifted bands that correspond to Fun30/Nucleosome complexes, this suggests that not only can a single Fun30 dimer complex bind the nucleosome but that two complexes can bind simultaneously. The quality of the Fun30/Nucleosome complex was considered sufficient for the preparation of a cryo-grid and subsequently images were captured via electron microscopy (Figure 5.7).

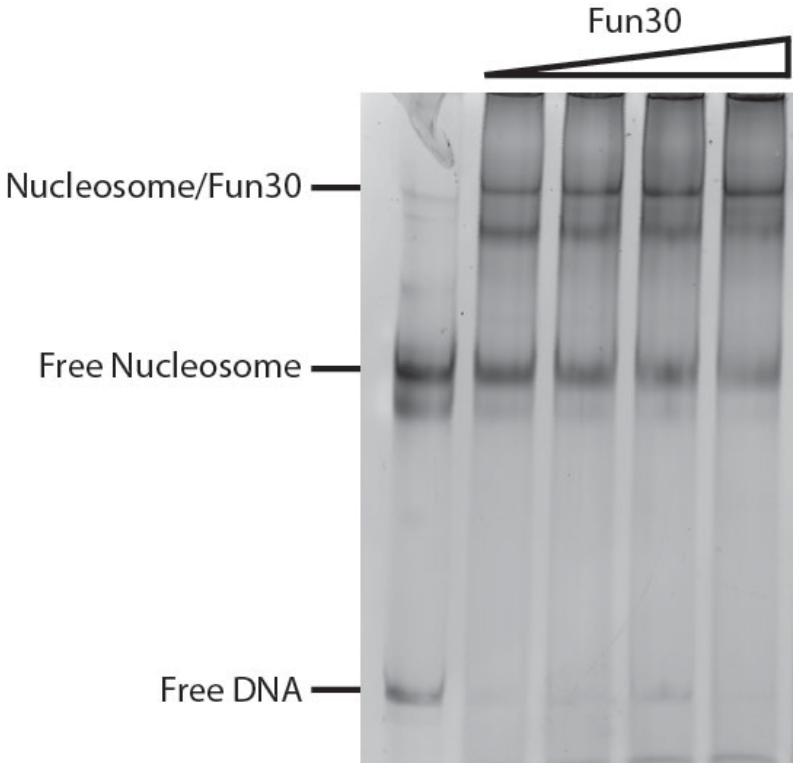

Figure 5.6. **Fun30 Nucleosome binding gradient.** Nucleosomes were assembled on the fragment 0W47 in which the 601 nucleosome positioning sequence directs assembly of a nucleosome such that it is flanked by 47 bp of linker DNA on one side. Incubation of 30 nM of 0W47 nucleosomes with increasing concentrations of Fun30 (28 nM – 1µM) resulted in gel shifted species (Nuc/Fun30) as highlighted on the figure.

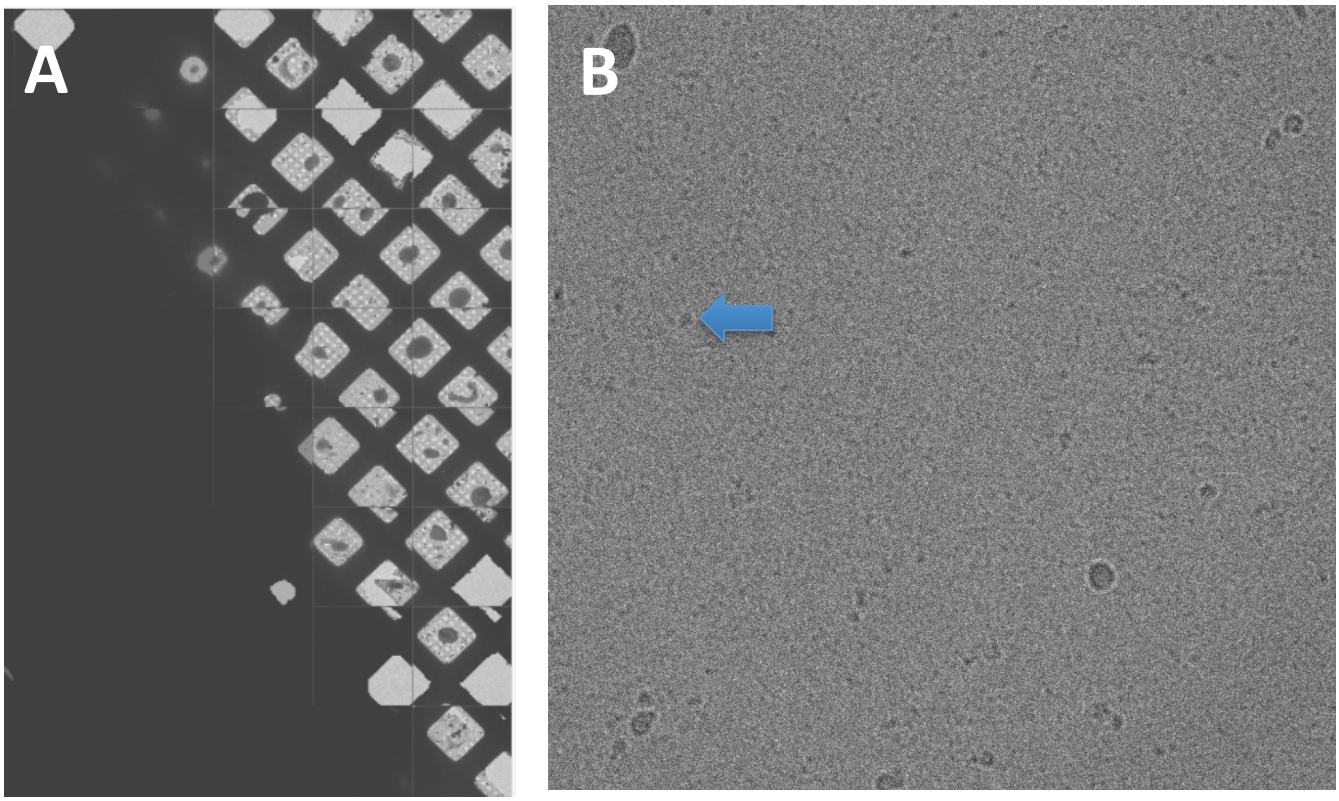

Figure 5.7.**Unprocessed images of the Fun30-Nucleosome complex by cryo-electron microscopy.** (A) Low-magnification image showing the ribbon of vitreous sections after electrostatic charging. (B) A higher-magnification image of the frozen-hydrated specimens of purified Fun30-Nucleosome complex. Complexes with distinct orientations relative to the electron beam may be discerned. Images captured by Dr. Ramasubramanian Sundaramoorthy (PhD).

5.2 *Chaetomium thermophilum* homologs of Fun30 and CHD1

5.2.1 Expression and Purification of CtFun30 and CtCHD1

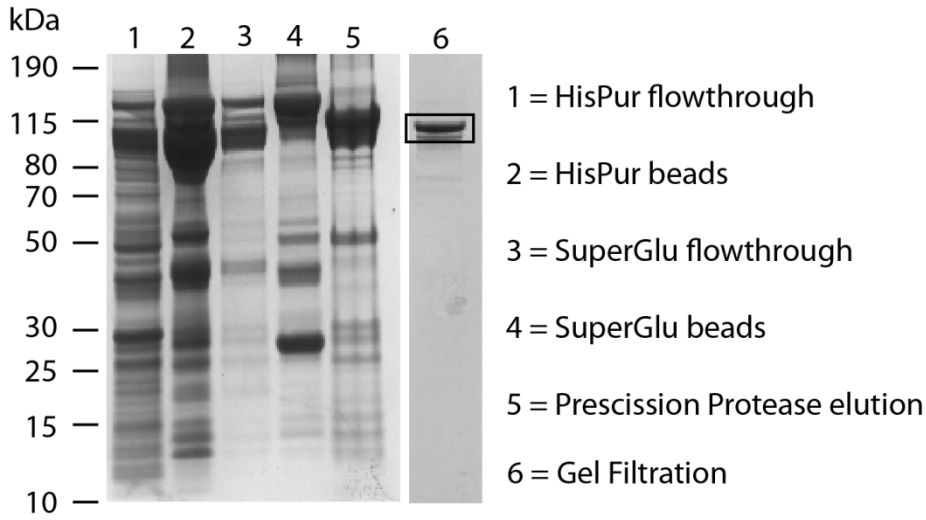

Figure 5.8. **Purification of recombinant CtFun30.** Nine litres of autoinduction media inoculated with CtFun30 was harvested and purified, 10µl samples were collected at six steps in the protocol as detailed in the figure legend and run on a 4-12% bis-tris gel. The final band of interest indicating CtFun30 after Gel Filtration (~110kDa) is highlighted in the figure. The final yield equalled ~200µl of ~3mg/ml protein.

Being large and dynamic proteins Snf2 family proteins are potentially difficult subjects for structural characterisation. Within other protein families it has been found that identifying homologs from thermophiles can provide a source of material better suited to structural characterisation (Freeman et al., 2014). In many cases this has involved the purification of orthologs from thermophilic archeabacteria. As the chromatin of archae is significantly different from that of euchareae we have instead identified Snf2 family proteins within the genome of the recently sequenced thermophilic yeast *Chaetomium thermophilum*. The *Chaetomium thermophilum* genome consists of thermophilic proteins which have

demonstrated better biophysical behaviour for use in structural investigation than their *Saccharomyces cerevisiae* homologs in studies of other proteins such as GEN1 (Freeman et al., 2014) and the Rvb1 and Rvb2 subunits of the Ino80 chromatin remodelling complex.

The *Chaetomium thermophilum* homolog of Fun30 (referred to as CtFun30) was obtained via gene synthesis and subsequently sub-cloned into a Pgex6P plasmid for expression. CtFun30 was found to be viable for expression in *E.coli* as well as being capable of expression and purification in autoinduction media allowing the protein to be produced in a similar manner as *Saccharomyces cerevisiae* Fun30 without the need to alter the protocol for their preparation in any significant manner. Further optimisation of the purification protocol has increased the purity of each subsequent CtFun30 purification.

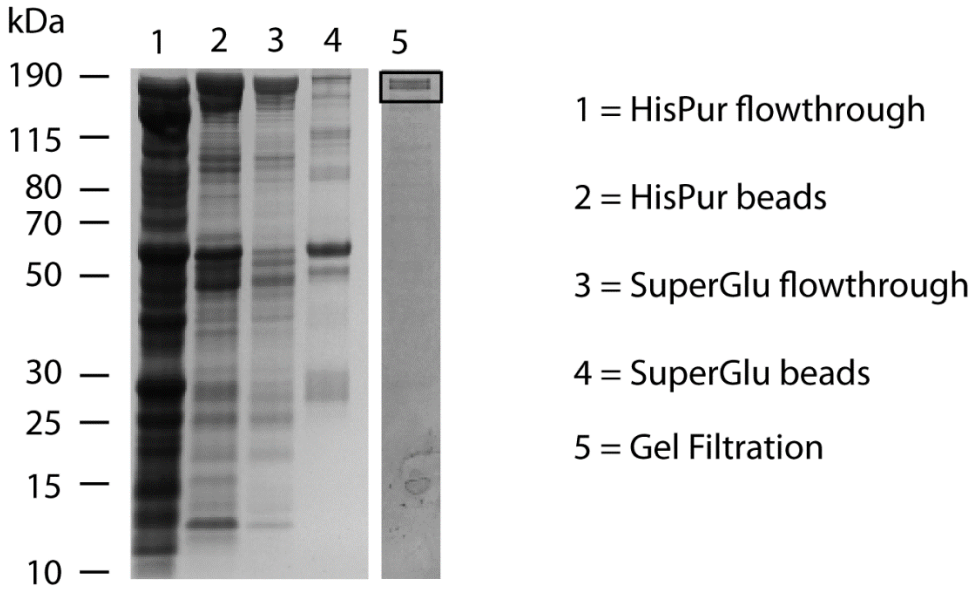

Figure 5.9. **Purification of recombinant CtCHD1.** Nine litres of autoinduction media inoculated with CtCHD1 was harvested and purified, 10µl samples were collected at six steps in the protocol as detailed in the figure legend and ran on a 4-12% bis-tris gel. The final band of interest indicating CtCHD1 after gel filtration (~200kDa) is highlighted in the figure. The final yield equalled ~120µl of ~5mg/ml protein.

The *Chaetomium thermophilum* homolog of CHD1 (referred to as CtCHD1) was also obtained via gene synthesis and subsequently sub-cloned into a Pgex6P plasmid for expression. CtCHD1 was found to be viable for expression in *E.coli* as well as being capable

of expression and purification in autoinduction media allowing the protein to be produced in a similar manner as *Saccharomyces cerevisiae* CHD1 without the need to alter the protocol for their preparation in any significant manner. A significant contaminant band can be seen after eluting from the SuperGlu beads at 60KDa indicating a glutathione-conjugated product smaller than CHD1. This could possibly be due to protease action on CHD1 leading to truncated CHD1 product. This contaminent is completely removed during the gel filtration stage.

5.2.2 ctFun30 and ctCHD1 Nucleosome Binding

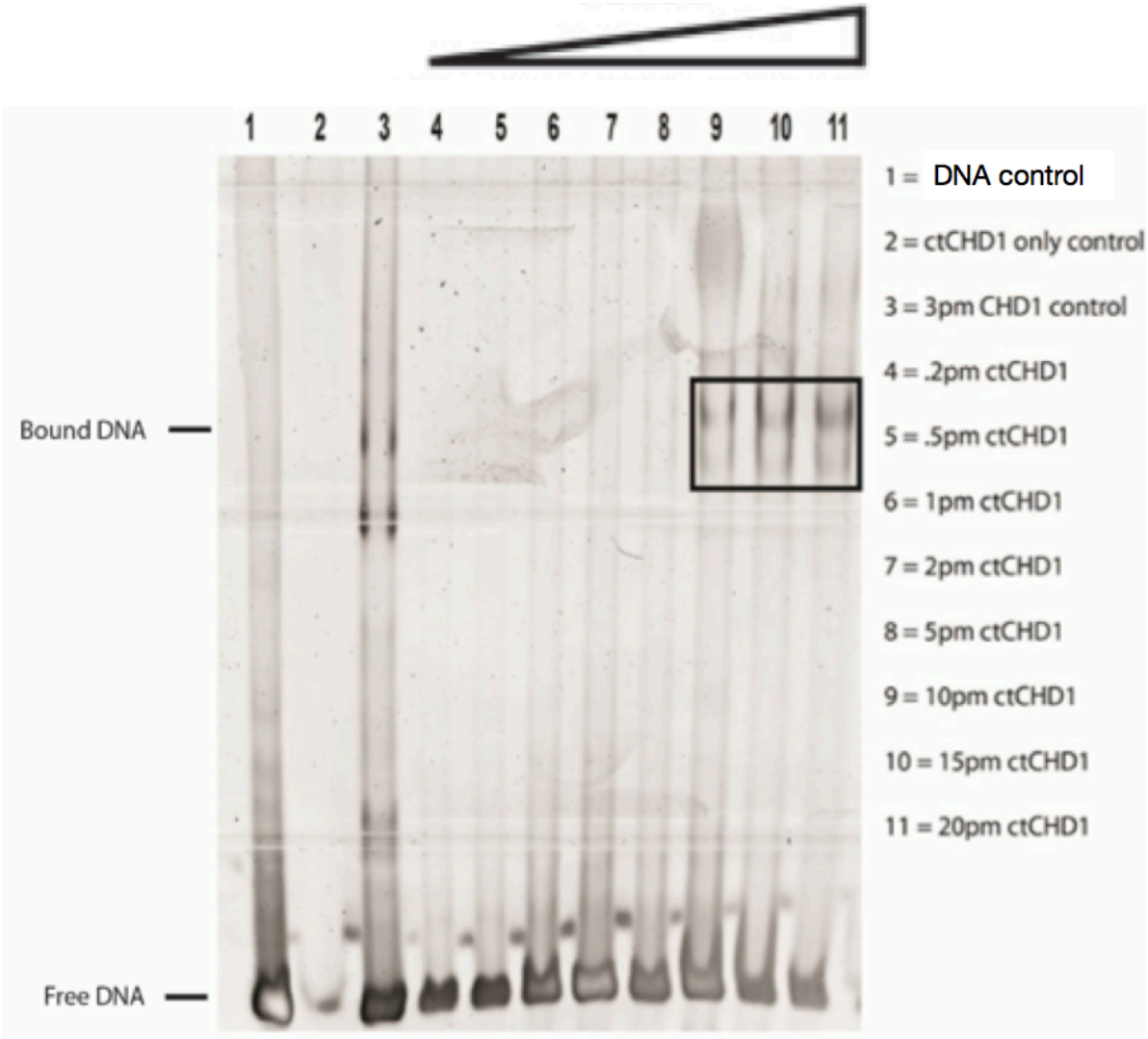

Figure 5.10. **CtCHD1 DNA binding assay.** 30nm of the DNA template 0W47 (a template in which the 601 nucleosome positioning sequence directs assembly of a nucleosome such that

it is flanked by 47 bp of linker DNA on one side) was incubated with increasing concentrations of CtCHD1 (.2pM – 20pM, lanes 4-11) resulting in gel shifted species (0W47/CtCHD1) as highlighted in the figure.

Chromatin remodelling complexes must be capable of recognizing their chromatin substrate in order to begin remodelling nucleosomes. Many chromatin remodelling complexes including *Saccharomyces cerevisiae* Fun30 and CHD1 have previously been shown to bind both DNA and Nucleosomes (Awad et al., 2010). The DNA binding ability of ctCHD1 was compared to that of *Saccharomyces cerevisiae* CHD1 (Figure 5.10). CtCHD1 was observed to bind DNA fragments as indicated by a gel shifted species of DNA/ctCHD1 but only at concentrations three-fold higher than the CHD1 control. Based on these results CtCHD1 was prioritised for structural studies.

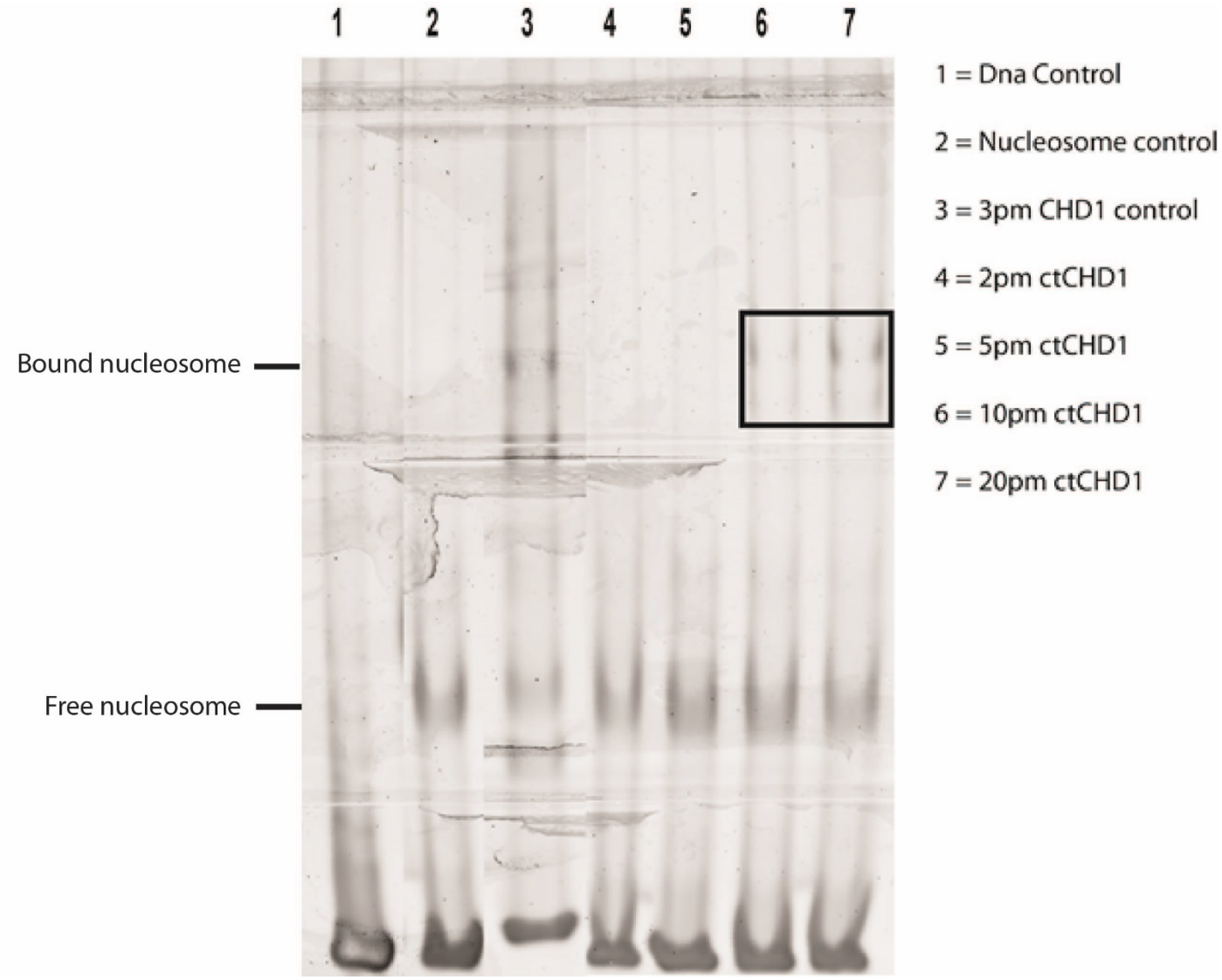

Figure 5.11. **CtCHD1 Nucleosome binding assay.** Nucleosomes were assembled on the fragment 0W47 in which the 601 nucleosome positioning sequence directs assembly of a nucleosome such that it is flanked by 47 bp of linker DNA on one side. Incubation of 30 nM of 0W47 nucleosomes with increasing concentrations of CtCHD1 (2pM – 20pM, lanes 4-7) resulted in gel shifted species (Nuc/CtCHD1) as highlighted on the figure.

The nucleosome binding ability of both ctFun30 and CtCHD1 were compared against *Saccharomyces cerevisiae* CHD1 (Figure 5.11). CtCHD1 demonstrated the ability to bind 0W47 nucleosomes as indicated by a gel shifted species of Nucleosome/CtCHD1 but only at concentrations three-fold higher than the CHD1 control which itself was a higher concentration than is necessary to observe binding.

6. Discussion

6.1 Expression of Fun30 and Fun30 constructs

Due to persistent issues with both the growth of competent *E.coli* cells and the expression of recombinant proteins in autoinduction media, significant yields of both Fun30 and the Fun30 mutants were limited during the initial stages of this project. These problems were attributed to the media due to similar problems occurring with the growth and expression of other chromatin remodelers within the lab and previous issues with autoinduction media predating the start of this project. As autoinduction media had previously been determined to give a better yield when expressing Fun30 in culture, when compared to other media that require induction of protein expression with IPTG (such as LB), it was the primary media used throughout this project. Attempts to address this problem included using larger starter cultures when inoculating large volumes of media, addition of trace elements to the media to potentially improve its robustness (Based on Studier. Protein Expression and Purification. 2005. 207-234) and preparing our own autoinduction media based on the Studier recipe. However these approaches were unsuccessful in improving yields of Fun30 and the Fun30 constructs. Increases in protein yields were observed over time most likely attributed to an increase in the quality of the provided media. These increased yields may also be a result of continued practise of the protocol over the weeks these expressions and purifications were carried out.

6.2 Purification of Fun30 and Fun30 constructs

The Fun30 constructs listed in Table. 1 were found to be viable for expression in *E.coli* as well as being capable of expression and purification in autoinduction media allowing these constructs to be produced in a similar manner as the full length molecule without the need to alter the protocol for their preparation in any significant manner. Improvements however can be made in the purification process by minimizing the amount of protein lost during each step in the protocol. A significant amount of Fun30 can still be seen bound to HisPur beads following elution (Fig. 5.3) indicating that more stringent elution with imidazole may

improve overall protein yields when using this method of purification. Loss of Fun30 can also be observed in the flow-through from SuperGlu beads indicating that some proportion of Fun30 is not successfully binding to the SuperGlu beads and is lost to the flow-through. Rebinding of the flow-through to SuperGlu beads is one approach that may decrease the loss of Fun30 at this stage of the protocol.

The chromatogram produced by gel filtration chromatography of the full length Fun30 sample shows the purified Fun30 sample eluting before a CHD1 sample eluting under the same conditions. As CHD1 is ~70kDa larger than the Fun30 monomer this suggests that Fun30 is purified as Homodimer of ~256kDa which is consistent with the literature. Investigation of the chromatograms produced by the Fun30 constructs when purified via gel filtration chromatography should allow more information on the dimerization domain of Fun30 to be obtained by determining which regions' deletion causes a loss of dimerization and subsequent elution of Fun30 as a monomer.

6.3 Cryo-electron microscopy of the Fun30-Nucleosome complex

Cryo-electron microscopy modelling can be particularly beneficial when combined with atomic models of either the complex or its sub-units. The primary disadvantage of this technique is the low-contrast nature of the images due to the differences in cross section between the macromolecule and its surrounding solution. This is of particular concern when studying a molecule with little to no structural information, as in the case of Fun30, where it becomes difficult to validate results obtained from image processing (Milne et al., 2013). The images obtained from the initial cryo-electron microscopy experiment are promising however as distinct, albeit low resolution complexes are detectable suggesting further optimisation of this experiment could yield better resolution images suitable for further processing.

6.3 Purification of CtFun30 and CtCHD1

CtFun30 and CtCHD1 were found to be viable for expression in *E.coli* as well as being capable of expression and purification in autoinduction media allowing these proteins to be produced in a similar manner as their *Saccharomyces cerevisiae* homologs without the need to alter the protocol for their preparation in any significant manner. This is notable as *Saccharomyces cerevisiae* CHD1 requires a C-terminal truncation to achieve optimal

expression whereas CtCHD1 is capable of being expressed as a full length protein. This is advantageous as it allows structural studies involving the C-terminal region that would not be possible with CHD1 to be carried out with CtCHD1. Further optimisation of the purification protocol has increased the purity of each subsequent CtCHD1 purification, from initial yields of 100µls of ~3.5 mg/ml CtCHD1 from an 8L culture to improved quantities of 100µls of ~6mg/ml obtained from the same volume of culture. These quantities are comparable to yields of *Saccharomyces cerevisiae* CHD1 from similar volumes. In the purification of CtCHD1 as seen in Figure 5.9 two closely spaced bands can be seen at sizes corresponding to CtCHD1. It is likely given the nature of the purification carried out that both of these bands are derived from CtCHD1 given their stepwise purification indicating they possess both the 6-His and GST tags required for purification using this protocol. It is possible that a subpopulation of CtCHD1 suffers some form of degradation during either the expression or purification of the protein and as such can be seen as a lower weight band on this gel. There may also be some form of post-translational modification occurring where the CtCHD1 molecule is modified by the addition of a new molecular group leading to a larger molecule that can be seen as a higher weight band on the gel, although it is unlikely that bacterial PTMs (post-translational modification) are affecting non-endogenous yeast proteins. Perhaps the simplest explanation is the possible loss of the N-terminal His-Tag, which is not normally lost during the purification process, the lower weight band could be attributed to the difference in weight between CtCHD1 including this tag and a subpopulation lacking the tag. Mass spectrometry of a purified CtCHD1 sample could determine if there is another sub population of CtCHD1 present in the sample and based on its size and composition the cause of this tandem band formation could be determined. Aside from the purification of a doublet, the purity of the these proteins is of a high quality suggesting they may be very well suited for structural studies.

6.4 CtCHD1 and CtFun30 DNA and Nucleosome Binding

The low level of DNA and nucleosome binding observed with CtCHD1 is discouraging for structural studies with this molecule. In the case of CtCHD1 a threefold difference in binding ability may not be significant as long as it is possible to prepare a fully bound complex, however the complete lack of binding by CtFun30 is a more pressing obstacle when

considering further study. The low nucleosome and DNA binding activity demonstrated by the *Chaetomium thermophilum* homologs could suggest differing structural features when compared to *Saccharomyces cerevisiae* Fun30 and CHD1. These differences would likely be relatively minor considering the sequence similarity between these proteins and their *Saccharomyces cerevisiae* homologs but would be significant enough to greatly reduce their efficacy in nucleosome binding. This would limit their utility as target molecules for understanding the mechanisms of action of related remodelers like Ino80 and Swr1 as structural features and mechanisms identified in these homologs may not be as well conserved in *Saccharomyces cerevisiae* remodelers. It is also a possibility that CtCHD1 and CtFun30 retain specificity for *Chaetomium thermophilum* histones or specific DNA sequences found in *Chaetomium thermophilum* nucleosomal DNA.

6.5 Future Work

The constructs and homologs that have been designed and produced for this project can be utilised in the future using a variety of techniques that would allow more structural knowledge on Fun30 to be obtained. Both proteins can be isolated with a high level of purity and in quantities suitable for crystallisation trials. As CtFun30 exists as a dimer it would be of a sufficient size for cryo-EM both in complex with the nucleosome and on its own. Unlike crystallography this technique does not require the preparation of crystals that diffract to high resolution. Advances in processing and detector technology have meant it is now possible to obtain structural information to 3.3 Å from proteins as small as 170 kDa (Bai, McMullan, et al., 2015; Bai, Yan, et al., 2015). The initial experiments with cryo-EM and Fun30 are promising for both the study of *Saccharomyces cerevisiae* Fun30 as well as the study of CtFun30 and CtCHD1 with this technique.

Cross-linking mass spectrometry is an emerging tool for the modelling of proteins and protein complexes. Specific residues with reactive side groups such as amino groups, thiols and carboxylic acids within a protein can be cross-linked using a suitable cross-linker such as disuccinimidyl suberate (DSS) provided the residues are within a certain distance of one another (Rappsilber, 2011) . The cross-linked protein can then be digested into peptides and using mass spectrometry the peptides that have been covalently coupled via the cross-linker

can be identified. This analysis can identify specific residues that were cross-linked to one another and therefore provide valuable structural data on the relative distance from one residue to another determined by the length of the cross-linker (Rappsilber, 2011) . The advantage of this technique is that relatively small amounts of protein are required and all potentially cross-linked residues can be studied in one experiment. No prior structural information is required to adopt the approach. Limitations are that it is difficult to identify cross-linked peptides from the large datasets collected, and that a relatively small number of useful constraints are obtained for each protein studied. Possible approaches that can simplify this data analysis include using cross linkers that are chemically fixed to a specific residue on one end, greatly reducing the number of possible cross-links as one residue in each potential cross-link is already known.

The utilisation of site directed spin labelling in combination with EPR (electron paramagnetic resonance) to investigate the structural dynamics of a protein is a well-established method that has been applied within our research group on other chromatin remodelers. EPR spectroscopy can measure the dipolar interaction between these spin labels and provide inter-nitroxide distance measurements between the two points (Czogalla et al., 2007) . This type of measurement is often referred to as a PELDOR (pulsed electron-electron double resonance) measurement. PELDOR experiments allows quantitative structural information on the labelled protein to be obtained, and with multiple measurements the overall protein topography, can be modelled (Czogalla et al., 2007) . EPR measurements provide an intermediate resolution range when compared to high-resolution techniques such as X-ray crystallography and low resolution techniques such as SAX (small angle X-ray diffraction). EPR however is most valuable when certain elements of the protein structure are already known to aid in the choosing of labelling sites and the processing of data provided by distance measurements. A CtCHD1 mutant lacking cysteine residues has been produced and expressed in useful quantities. This mutant can potentially act a starting point for the introduction of engineered cysteine mutations at strategic locations for attachment of paramagnetic spin labels. These would provide a means of investigating the structure of this protein by EPR. CtCHD1 presents a useful target for EPR experiments due the larger body of structural information available for CHD1 when compared to Fun30 allowing sites for spin-labelling to be more easily selected. In addition, Fun30 contains 7 native cysteines and Ctfun30 contains 15, the loss of these cysteines may have detrimental effects on the protein's

activity and folding. To assess this it will be important to test the activity of the cysteine-free proteins.

SAX experiments are also currently feasible as the proteins to be studied can be purified on a mg scale. SAX is advantageous when compared to X-Ray Crystallography as crystallization of the protein is not required however SAX experiments produce low resolution data that is best utilised in conjunction with other higher resolution techniques. SAX and EPR can be used in the future to give structural data on Fun30 when interacting with other molecules specifically the nucleosome. This approach could give insights into the transitional complex Fun30 forms with the nucleosome during the process of chromatin remodelling.

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List of Abbreviations

FUN30 - Function Unknown Now 30

CHD1 - Chromodomain Helicase DNA Binding Protein 1

HAT – Histone Acetytransferase

HDAC – Histone Deacetyltransferase

HP1 - Heterochromatin Protein 1

UIM – Ubiquitin Interacting Domains

UBA – Ubiquitin associated Domains

CUE – Coupling of Ubiquitin to ER degradation

HMG – High Mobility Group

CAF-1 – Chromatin Assembly Factor 1

NAP1 – Nucleosome Assembly Protein 1

INO80 – INOsitol Requiring

SWR1 – Swi2/Snf2 related

ARP4 – Actin Related Protein 4

ETL1 – Enhancer Trap Locus 1

SMARCAD1 - SWI/SNF-related, Matrix-associated, Actin-dependent Regulator of Chromatin, containing DEAD/H box 1

HMR - Hidden MAT Right

TSS – Transcription start Site

LTR – Long Terminal Repeat

SAGA – Spt-Ada-Gcn5 acetyltransferase

SLIK – SAGA Like

SANT - Switching-defective protein 3 (Swi3), adaptor 2 (Ada2), nuclear receptor co-repressor (N-CoR) and transcription factor (TF)IIIB

SLIDE - SANT-like ISWI

Cryo -EM – Cryo Electron Microscopy

SAX – Small Angle X-ray Scattering

EPR – Electron Paramagnetic Resonance

PELDOR – Pulsed Electron-Electron Double Resonance