Investigating how post translational modifications of p97 affect its function and regulation during DNA synthesis

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Abstract

p97 is a AAA+ ATPase with very diverse functions, from DNA replication to proteolysis. However, it is unknown how p97 is regulated throughout the cell cycle to affect the variety of its substrates differently in the right place and at the right time. In DNA replication, it is involved in removing the helicase from the chromatin when replication has ended. A previous member in the lab, discovered that p97 has different post translational modifications in S and M phases of the cell cycle including phosphorylation and ubiquitination. His-tagged mutant p97 proteins were made that were either catalytically dead, phospho-dead, phospho-mimicking or ubiquitin dead. These were then tested in the cell free *Xenopus* egg extract to see how they affect the protein dynamics of the replication machinery components: Mcm7, PCNA, Psf2 and Cdc45 during DNA replication via chromatin isolations. The interactions of these mutants with some of p97's cofactors were also studied via His-pulldowns and it was found that in S-phase, phosphorylation could increase interaction of p97 with Npl4 and p47, while in M-phase, it may also increase with p47 and Ufd1. When p97 is catalytically dead, it may increase interaction with Ufd1. Alongside this, RPA and WRN were identified as proteins that may interact with p97 during DNA replication.

Acknowledgements

Firstly, I would like to express my immense gratitude towards my supervisor, Dr Agnieszka (Aga) Gambus, who saw my potential and allowed me a space in the laboratory to share her passion in the subject. The countless questions that were answered by her increased my confidence in my project.

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Thank you to my best friend Annika, her sister Freeha and her family who supported me through this intense year and never failed to make me laugh, even after a hard day with the experiments.

Finally, the biggest appreciation and thanks to my wonderful parents and grandparents who have nurtured me into the woman I am today both professionally and personally.

Dedication

For those who have made me who I am today:

My parents

and

grandparents

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1. Introduction

1.1. Ubiquitin

Ubiquitin is an 8.6 kDa protein made up of 76 amino acid residues, that can be attached to another protein (post translationally modifying the protein). This impacts a variety of cellular functions including cell cycle checkpoints, endocytosis and proteasomal degradation (Herhaus and Dikic, 2015, Hershko and Ciechanover, 1998, Kirkin and Dikic, 2007, Narasimhan et al., 1996). As it is such a vital protein in the body it has been widely researched, and it was shown that dysfunctional ubiquitylation can be linked to a range of diseases such as inflammation and cancer. This led to drugs targeting ubiquitylation processing to be produced, such as Bortezomib to treat cancer (Hoeller et al., 2006).

1.1.1. Structure

Ubiquitin's core contains α-helixes and β-sheets which are held tightly together by many hydrogen bonds between its residues (Vijay-Kumar et al., 1987). It also contains a β1/β2 loop with a leucine 8 (Leu8) residue that can adopt different shapes and is recognised by target proteins and binds to them (**Figure 1 b**). There are also hydrophobic regions on the ubiquitin's surface which have important interaction roles. The Isoleucine 44 (Ile44) and Ile36 patches control ubiquitin molecules interacting with each other. The phenylalanine 4 (Phe4) patch binds to ubiquitin-specific proteases and deubiquitinates (DUBs), while the TEK-box is involved in mitotic degradation of the ubiquitylated proteins. The C-terminus is also very important as its glycine 76 (Gly 76) residue binds to a lysine residue on a substrate via an isopeptide bond (**Figure 1 c**) (Conway de Macario and Macario, 2007, Komander and Rape, 2012, Ronau et al., 2016).

Another important feature of ubiquitin is its seven lysine (Lys, K) residues, which is where other ubiquitin molecules can bind to each other to form polyubiquitin chains. K48 is the most common linkage with more than half of the ubiquitin chains having this link (Chen and Sun, 2009). This signals for the ubiquitylated substrate to be degraded. Other K residues include 6, 11, 27, 29, 33 and 63 (**Figure 1 d**). Different types of chains send different signals to effector proteins to carry out different functions such as the K63-linked ubiquitin chain, which is involved in activating kinases and in deoxyribose nucleic acid (DNA) repair signalling (Chen and Sun, 2009, Liu et al., 2018). Ubiquitin also contains residues that can be used to post-translationally modify itself via adding phosphate groups (phosphorylation) on residues such as serine 57 (Ser57), or acetyl groups (CH3CO) (acetylation) on all but one of the K residues, thus, ubiquitin may be regulated in different ways (Swatek and Komander, 2016).

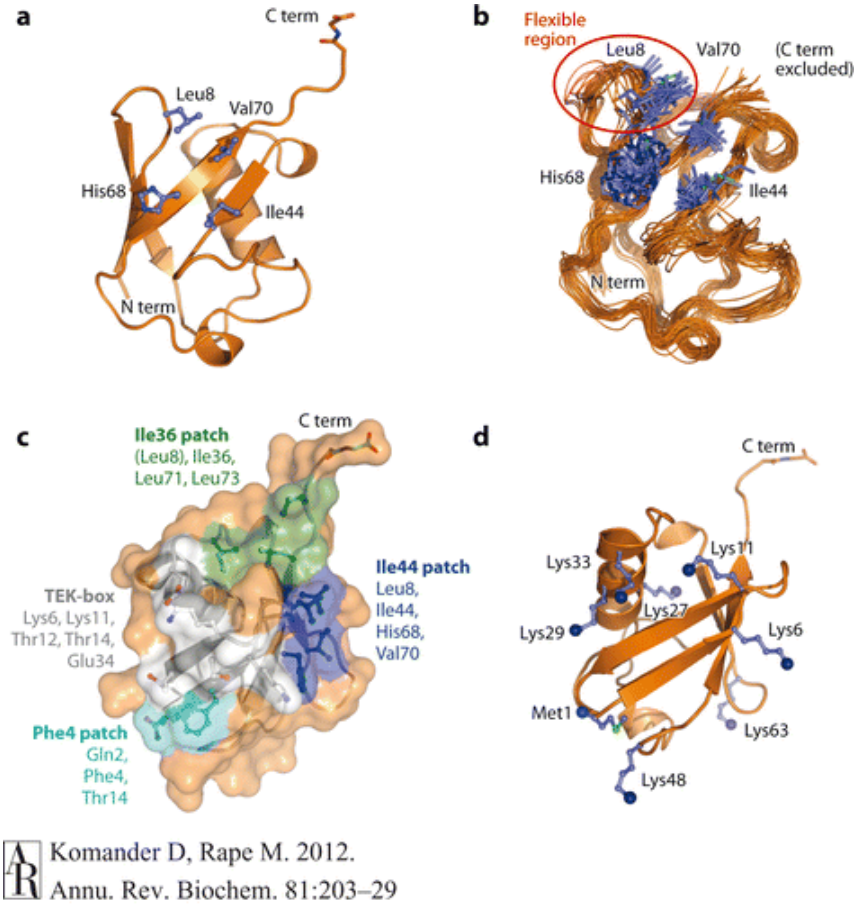


Figure 1: The structure of ubiquitin with different parts highlighted in different diagrams. **A** with the core, N and C terminus. Highlighted amino acids include Leu8 which is involved in substrate binding and Ile44 involved in the interaction between other ubiquitin molecules. **B** showing the structure created from several Nuclear Magnetic Resonance (NMR) tests with the flexible region highlighted with a red circle. **C** is the surface structure with the Ile44 patch, Ile36 patch, Phe4 patch and TEK-box highlighted. **D** shows the amino acids involved in binding other ubiquitin molecules indicated by the blue circles (Komander and Rape, 2012)

1.1.2. Ubiquitylation Cascade

The ubiquitylation cascade is a multi-step pathway that is present in eukaryotes. It involves a series of enzymes that attach ubiquitin to a substrate. To add the ubiquitin chain, three enzymes

are used: an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2) and an ubiquitin ligase (E3) (Gong et al., 2016).

Firstly, the ubiquitin needs to be activated which happens when the ubiquitin's C-terminus Gly residue binds to a cysteine (cys) residue on the E1 enzyme, via a thioester bond, creating an enzyme-bound ubiquitin adenylate intermediate. Adenosine triphosphate (ATP) is required for this to occur, and Adenosine monophosphate (AMP) and PP_i is released. Next, the ubiquitin binds to the E2's Cys residue via transacylation. The E3 enzyme recognises the substrate, specifically the ϵ-amino group lysine residues, which then binds to the C-terminus of the ubiquitin via an amide isopeptide bond, resulting in ubiquitylation. If a polyubiquitin chain is to be formed, once the first ubiquitin is attached to the substrate, other ubiquitin molecules can bind to the K residues in ubiquitin via their C-terminus (**Figure 2**) (Gong et al., 2016, Hershko and Ciechanover, 1998, Narasimhan et al., 1996).

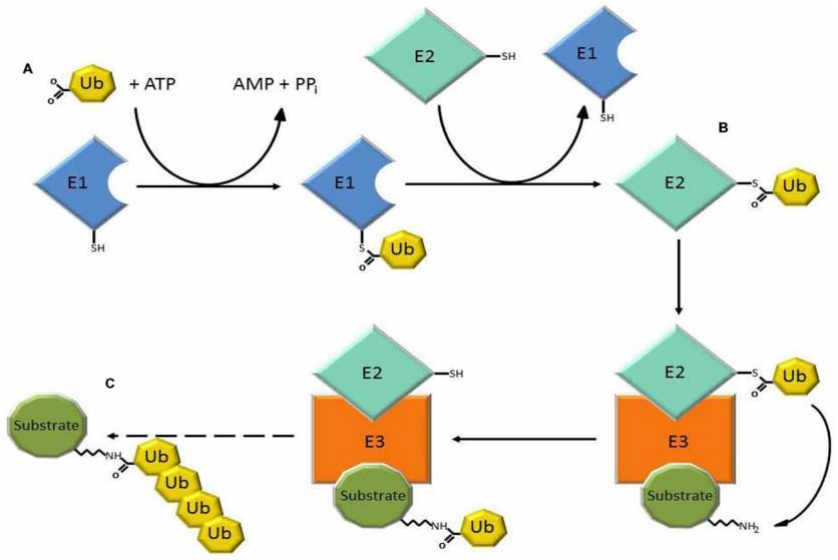


Figure 2: The steps of the ubiquitylation cascade showing that first (**A**) the ubiquitin is attached to the E1 enzyme (using energy released from ATP), this then attaches to the E2 enzyme (**B**). The E3 enzyme then attaches to the E2 enzyme and to the substrate. This stimulates attachment of the ubiquitin to the substrate. If required, more ubiquitin molecules can be attached to form a polyubiquitin chain (**C**) (Gong et al., 2016)

1.1.3. Ubiquitin-Proteasome System (UPS)

The Ubiquitin-Proteasome System (UPS) is the degradation of proteins with K11, K29 and K48 polyubiquitin chains attached (Bard et al., 2018). Once the protein has the polyubiquitin chain attached, a 26S proteasome complex recognises the chain and degrades the ubiquitylated protein using energy from the hydrolysis of ATP. A 26S proteasome complex is an enzyme that is an ATPases Associated with diverse cellular Activities (AAA)+. An AAA+ ATPase is a protein that aids the breakdown of ATP via hydrolysis and uses the energy released to carry out its mechanical function (Miller and Enemark, 2016) . It is made up of two parts. Firstly, a 20S core that is a hollow barrel shape which disassembles proteins by pulling their peptides

through the hollow core (using ATP). Secondly, is the 19S regulatory structure that sits on one or both ends of the core, it recognises the protein and feeds it through the core (**Figure 3)**. Once the peptide has been pulled through, the protein is broken into small peptides and can no longer carry out its function (Bard et al., 2018, Hershko and Ciechanover, 1998).

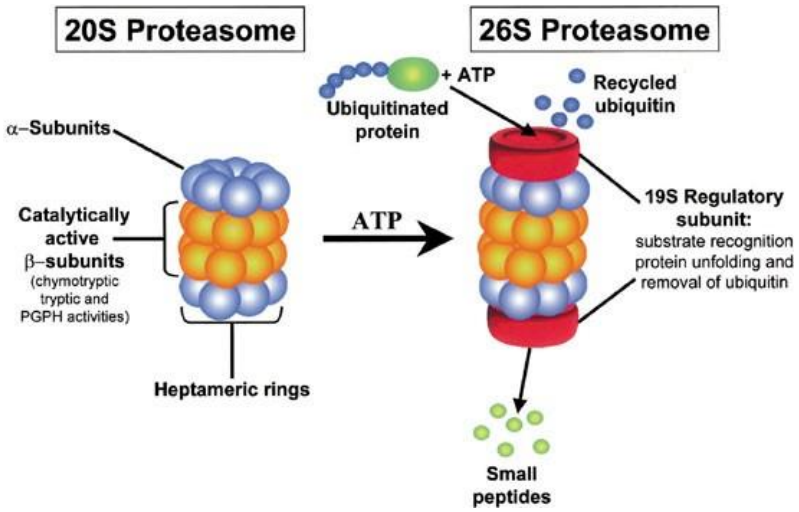


Figure 3: A diagram of a 26S proteasome with the 20S subunit containing the catalytic core sitting between the regulatory subunits (19S) on either end (Almond and Cohen, 2002)

1.2. p97

p97 is a 97 kDa protein that has many different names including Valosin-containing protein (VCP) in humans, in yeast it is known as cell division control protein 48 (CDC48) and in *Drosophila* as TER97 (Wang et al., 2004). p97 is highly concentrated in cells (1% of all cellular proteins) and works by recognising polyubiquitinated proteins, unfolding them and often passing them to a proteosome. This results in the protein being able to be broken down further as shown in **Figure 4**. As p97 regulates many different proteins, it has many different roles in different processes such as DNA replication, autophagy and proteolysis (Kloppsteck et al., 2012, Yamanaka et al., 2012)

1.2.1. p97 and UPS system

p97 is an important part of the UPS system as it acts as a chaperone to transfer the polypeptide chain to a 26S proteasome, to breakdown the chain further (**Figure 4**) (Kloppsteck et al., 2012).

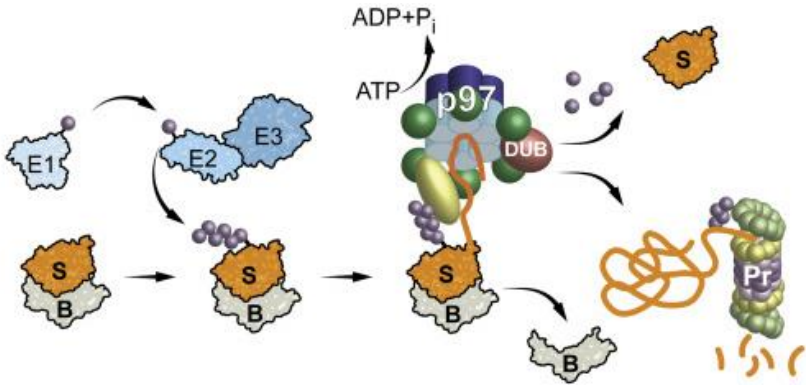


Figure 4: p97's role in UPS. Ubiquitin binds to the E1 enzyme, then to E2. E3 then binds to E2 which adds the ubiquitin to the protein and a ubiquitin chain can then be made. p97 recognises the polyubiquitin chain attached to the protein and unwinds the substrate into its amino acid chain. This is then passed to a proteosome to be degraded further (Van den Boom and Meyer, 2018)

1.2.2. Structure of p97

p97 is a class 2 AAA+ ATPase enzyme (Wang et al., 2004) which is formed of six subunits that are all identical and come together to form a ring structure. It is in the class 2 due to the fact that it has two ATPase domains that work together (Xia et al., 2016). Each subunit has two ATPase domains (D1 and D2) which are highly conserved. The D2 domain is vital for its function as when this is mutated, ATP cannot be hydrolysed. The D1 domain acts as a support for the D2 domain and is involved in the heat-induced ATPase activity. When heated, the D1 ring enlarges which makes it more available for ATP to bind and to be hydrolysed (Bastola et al., 2017, Beskow et al., 2009, Song et al., 2003, Yamanaka et al., 2012).

p97 contains several domains: the N-terminus domain which is formed from amino acids 1 to 187 in human p97 protein, the N-D1 linker from 188 to 208, D1 from 209 to 460, the D1-D2 linker 461 to 480 and D2 481 to 761. The six p97 molecules then come together to form a 'mushroom shape' with the N and N-D1 linker domains at the top (cap) and the D1 and D2 domains at the bottom (stalk) (**Figure 5**). The D1 and D2 domains are very similar with only a few subtle differences. They are both constructed from α-helixes and β-sheets with a α/β fold at the N-terminus. However, some of the α-helixes are arranged slightly differently between the domains e.g. in the D1 domain the α12 is tilted down (Wang et al., 2004). p97 also can be post-translationally modified in numerous ways such as by ubiquitination, phosphorylation and acetylation (Hänzelmann and Schindelin, 2017).

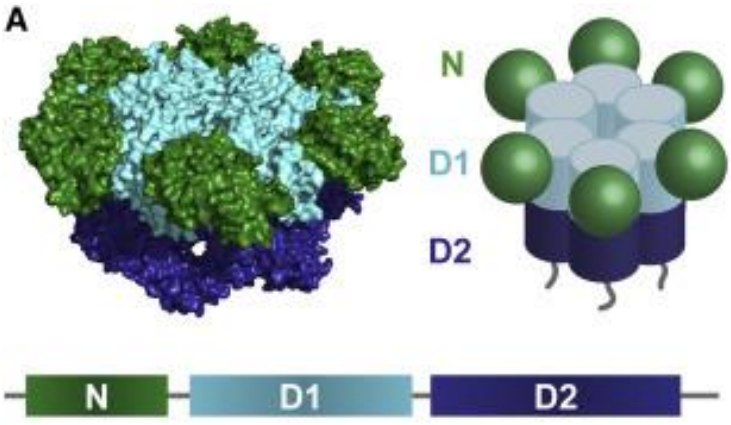


Figure 5: The organisation of a single subunit of p97 is shown at the bottom of the image with the N, D1 and D2 domains shown. These then come together to form the mushroom shape. Top right image showing how this come together. The top left is a side view of the 3D structural model (Van den Boom and Meyer, 2018)

1.2.2. How p97 works

1.2.2.1. Cofactors

For p97 to recognise its various substrates, cofactors (p97 adaptors) need to bind at either the N or C-terminus of the 3D hexametric structure. So far, there are about 30 cofactors that have been shown to interact with p97 and these are separated into three main classes: substrate-recruiting cofactors which bind to substrates and to p97 e.g. ubiquitin-associated and ubiquitin-regulatory X (UBA-UBX) domains containing cofactors. The second main class is substrate processing cofactors that process ubiquitin chains attached to substrates e.g. DUBs. Finally type three are regulatory cofactors that could recycle different parts of p97 e.g. UBXD4 (Hänzelmann and Schindelin, 2017).

Cofactors can also be grouped into major and minor. The major cofactors facilitate p97's activity and are related to a specific cellular process. For example, for the chromatin functions of p97, Ubiquitin Recognition Factor 1 (Ufd1) and Nuclear Protein Localization 4 (Npl4) form a dimer of major cofactors which facilitate p97 interactions with chromatin substrates. The minor cofactors are specific for the substrate that p97 needs to interact with such as Fas Associated Factor 1 (Faf1) and UBX domain protein 7 (Ubxn7) which are required for dissembling of the replisome (Tarcan et al., 2022).

p97 has many other cofactors which are involved in its other functions. An example is p47 which is a 47 kDa protein and is involved in Golgi cisternae regrowth and endoplasmic reticulum associated degradation (ERAD) (Kondo et al., 1997, Park et al., 2017).

1.2.2.2. Unfolding proteins

First, as **Figure 6** shows, the major and minor cofactors bind to p97 at the N-terminus. The cofactors recognise the polyubiquitin chain on a substrate (which has to be at least the length of five ubiquitin molecules long). The ubiquitin chain then starts to move through the catalytic core of the p97 complex. The chain first reaches the D1 domain, and when the hydrolysis of ATP has occurred, it moves it through to the D2 domain. This action allows the passing of the ubiquitin chain through the core. As it is attached to the substrate protein, it also pulls the substrate through the core thus unwinding the substrate's tertiary structure into its polypeptide chain (primary structure). Once the ubiquitin has reached the other side of p97, a DUB cuts the ubiquitin chain away which then releases it and can be reused. The substrate can then be delivered to a 26S proteosome to be degraded if required (Bodnar and Rapoport, 2017).

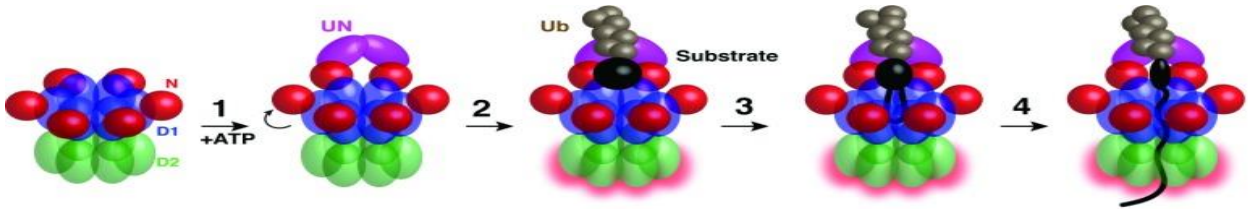


Figure 6: An overview of how p97 attaches and unfolds polyubiquitinated proteins. Firstly, with the aid of ATP, its cofactors (UN) bind to the N-terminus (**1**). Then a substrate with a polyubiquitin chain attaches (**2**) and the substrate is pulled through p97's core (**3**). This results in the substrate being broken down into its polypeptide chain (**4**) (Bodnar and Rapoport, 2017)

1.3. DNA replication

1.3.1. Overview

DNA is present in all living organisms and has the code for all instructional information for a cell. It is made up of four nitrogenous bases which are split into two groups. The first group being purines which have two cyclic rings. The purines are Adenine (A) and Guanine (G). The second is called pyrimidines which have one cyclic ring. The pyrimidines are Thymidine (T) and Cytosine (C) (Hartman, 1970). These are joined together via a phosphodiester bond in the backbone and complementary bases pair up to form the two complementary strands (Sinden, 1994).

For cells to divide for the growth of an organism, the DNA needs to be replicated. This occurs during the synthesis phase (S-phase) of the cell cycle and involves a high level of coordination between many different proteins (Laskey et al., 1989). The three main steps of DNA replication are initiation (loading proteins onto the chromatin), elongation (creating the complementary strand) and termination (stopping replication) (Dewar and Walter, 2017, Sclafani and Holzen, 2007).

One of the key proteins during replication is the helicase which unzips the DNA. In eukaryotes, the inactive core of the helicase is called the minichromosome maintenance complex 2-7 (Mcm2-7). Mcm2-7 binds to the chromatin at the origins of replication during growth 1 phase (G1-phase) of the cell cycle. Origins are marked by Origin Recognition Complexes (ORC). Cell Division Cycle 6 protein (Cdc6) and chromatin licensing and DNA replication factor 1 (Cdt1), help to load Mcm2-7 onto origins and form the pre-replicative complex (pre-RC). Next, during S-phase, Cell division control protein 45 (Cdc45) and GINS complex bind to Mcm2-7 to activate it by forming the Cdc45-Mcm-GINS (CMG) complex. Once activated, it unzips the

DNA to create a replication fork with two single strands of DNA (leading and lagging). Replication protein A (RPA) is another important protein in this step as it binds to exposed single stranded DNA (ssDNA), which stops the forks collapsing, and also assists the helicase in unwinding the DNA. This process occurs at many different places across the chromatin as there are many origins of replication. There are also many other proteins that are involved and some are shown in **Figure 7** below. (Bell and Dutta, 2002, Branzei and Foiani, 2010, Douglas et al., 2018, Wold and Kelly, 1988).

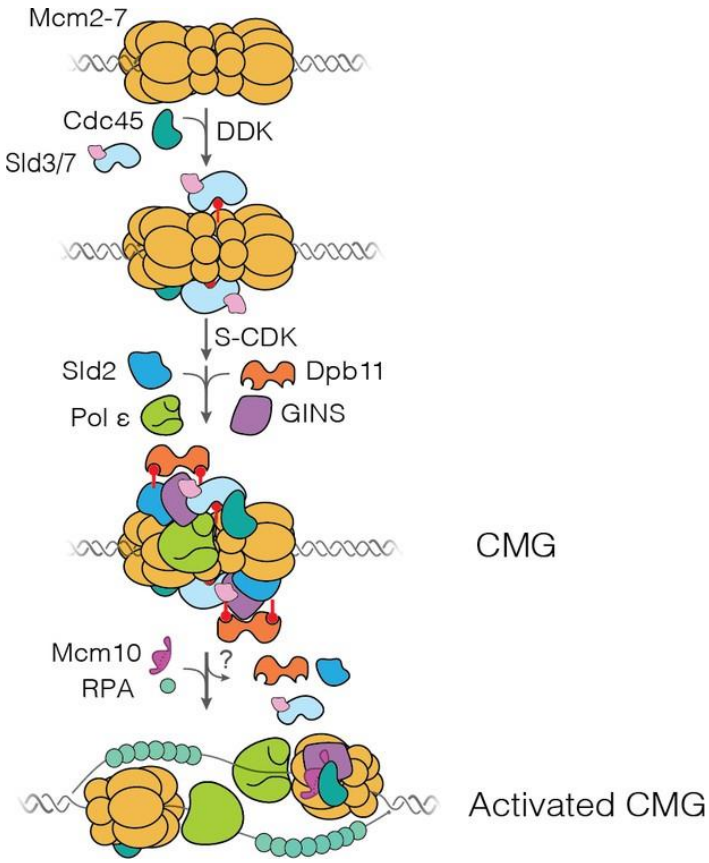


Figure 7: In pre-initiation, the MCM 2-7 complex bound to the DNA at the ORC with other proteins including Cdc45. Then other proteins bind such as RPA and GINS which initiate DNA replication. This is a model from S.cerevisia and the protein names are yeast and not from higher eukaryote (De Jesús-Kim et al., 2021)

Once the CMG helicase has been activated, many other proteins bind to the chromatin such as Polα/primase which produces short Ribonucleic acid/DNA (RNA/DNA) primers and DNA polymerases (pol). The pol's recognise the primers, read the template strand and makes a complementary DNA strand. The Pol can only make DNA in one direction (3' to 5'), however, DNA is antiparallel, therefore, the leading strand is made by polymerase epsilon (Polε) continuously. The lagging strand is made by polymerase delta (Polδ) in short fragments, which are known as Okazaki fragments. As there are gaps present between the fragments, they need

to be filled. This is achieved by first removing the RNA primer by Flap endonuclease 1 (FEN1) and Polδ. The gap is then filled by Polδ. As the new DNA strand is being synthesised, the Okazaki fragments need to be joined together via a phosphodiester bond, which is carried out by DNA ligase (Al-Behadili et al., 2018, Boehm et al., 2016, Chen and Sun, 2009, Dohrmann et al., 2011, Frick and Richardson, 2001, Johnson et al., 2015, Matsunaga et al., 2003). The model of organisation of these proteins is shown in **Figure 8**.

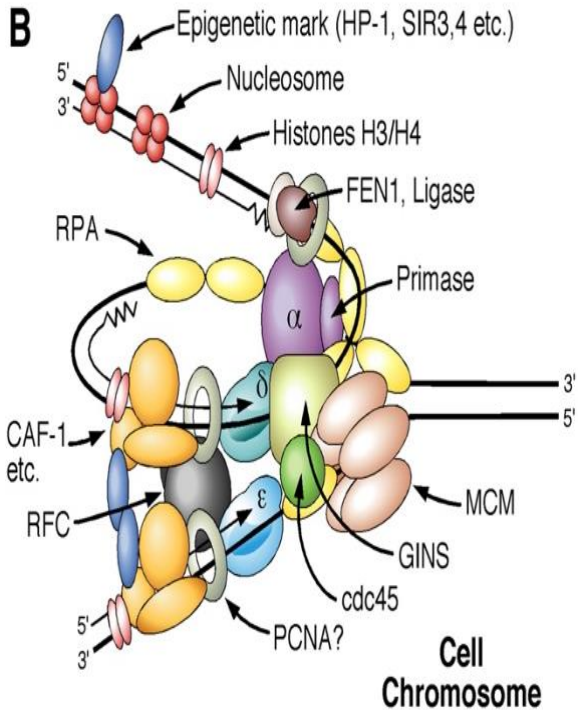


Figure 8: DNA synthesis. The two strands of DNA being separated via the active helicase consisting of Mcm2-7, GINS and Cdc45 subunits. Primase then produces a primer and pol ε reads the leading strand, while pol δ reads the lagging strand and create complementary strands. Proliferating cell nuclear antigen (PCNA) and RPA sit near the polymerases and aid their function. The processing of Okazaki fragments is carried out by enzymes such as FEN1 and Ligase1, which joins the backbone together to form one continuous strand (Stillman, 2008)

Once two replication forks originating from neighbouring origins come together, replication needs to be terminated, which involves the removal of the replisome proteins. DNA can become supercoiled during replication and form structures called catenanes. These are removed via decatenation. There are different ways this can be resolved. One way involves rotating one of the replisomes clockwise to form a pre-catenane behind the fork, which topoisomerase II (Topo II), can then come along to detangle the sister chromatids. The two converging helicases bypass each other and when they reach the end of the last Okazaki fragment, they need to be taken off the DNA which is done via the ubiquitination of the CMG complex (as previously mentioned), **Figure 9** (Dewar and Walter, 2017).

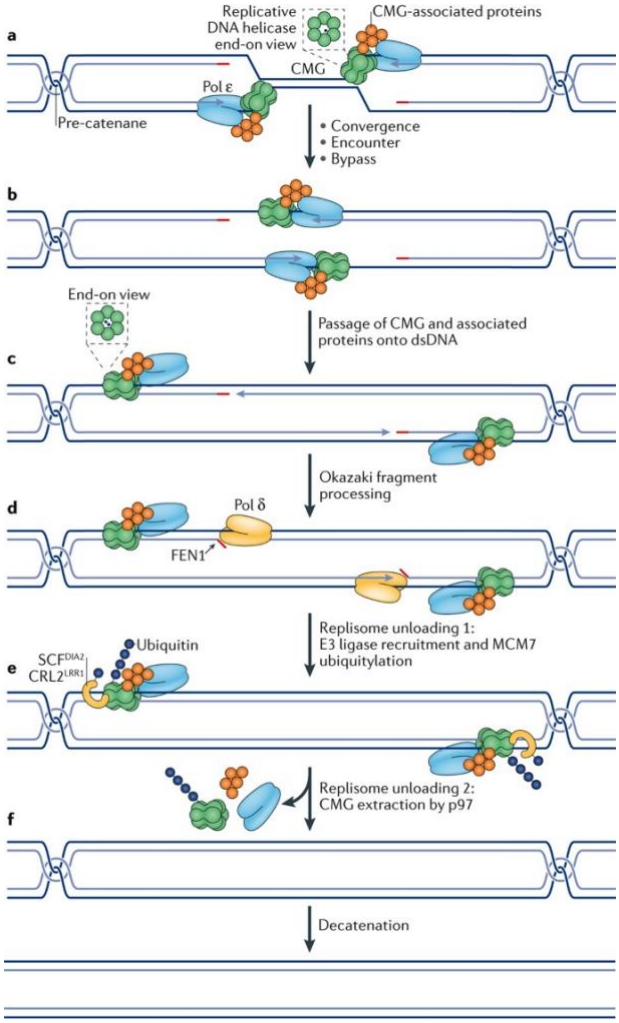


Figure 9: DNA replication termination in eukaryotes. In **A,** the two opposite helicases coming together and **B is** when they pass each other. Then in **C** the gaps between the Okazaki fragments are joined together. **D** is showing that polymerase are de-coupled from the helicase then in **E** is when the polyubiquitin chain is added to the helicase. Then in **F** is when the helicase dissociates from the replisome and finally decatenation occurs (Dewar and Walter, 2017)

1.3.2. Replication stress

Replication stress occurs when there is any perturbance to the DNA replication. This could be from DNA damage caused by various sources such as ultraviolet (UV) light or cellular metabolites (Gaillard et al., 2015). For example, when the UV is absorbed by the skin, the

photons change the electron energy in the pyrimidines. This leads to the purine and pyrimidines (A-T or G-C) to unbind and the adjacent pyrimidines (C and T) binding to each other resulting in a cyclobutene pyrimidine dimer (CPD). These cause changes in the double helix structure so can slow or stop DNA replication occurring after the mutation (Cadet and Douki, 2018).

When polymerases are blocked, helicase can sometimes continue to unwind the DNA. This results in longer stretches of ssDNA being present. DNA pol can be stalled for a number of reasons such as: mismatched nucleotides which need to be changed or the formation of different structures e.g. G4 quadruplexes from G-C rich stretches. Helicases can also be stalled in areas which are difficult to access e.g. heterochromatin where the DNA is wound tighter (Zeman and Cimprich, 2014). When long stretches of ssDNA are present, RPA binds to the ssDNA. This then activates a replication stress response. As a result, other proteins such as the ataxia telangiectasia mutated (ATM) and Rad3-related (ATR) bind. (Weisshart et al., 2004, Zeman and Cimprich, 2014). Another protein that is involved in DNA stress is Werner (WRN), which is thought to interact with RPA. Although the exact mechanisms are unclear, it has been suggested that WRN may switch places with RPA on the DNA (during stress) but they stay bound together, causing WRN to unwind the DNA, which allows other proteins to bind and repair the DNA (Brosh and Bohr, 2007).

This response can occur locally (in one replisome) or globally (across the whole cell nuclear DNA). As a result cells can respond in different ways to the damage causing a variety of outcomes, as shown in **Figure 10**. When the damage can be repaired, then synthesis can carry on as normal, however, if this is not the case the replication fork may need to be rescued by the neighbouring fork approaching from the opposite direction. The potential outcomes are shown

in **Figures 10 b** and **c**. During fork rescue (**Figure 10 b**) repriming or repriming could occur. During fork collapse (**Figure 10 c**) replisome migration or replication run off may occur. These can occur if ATR is not present during DNA stress or if there is a double strand break (DSB). A DSB can be harmful to the cell and can have serious consequences as it can cause mutations and can rearrange the order of the chromosomes to such an extent that it can lead to cancer or cell death (Cannan and Pederson, 2016, Zeman and Cimprich, 2014).

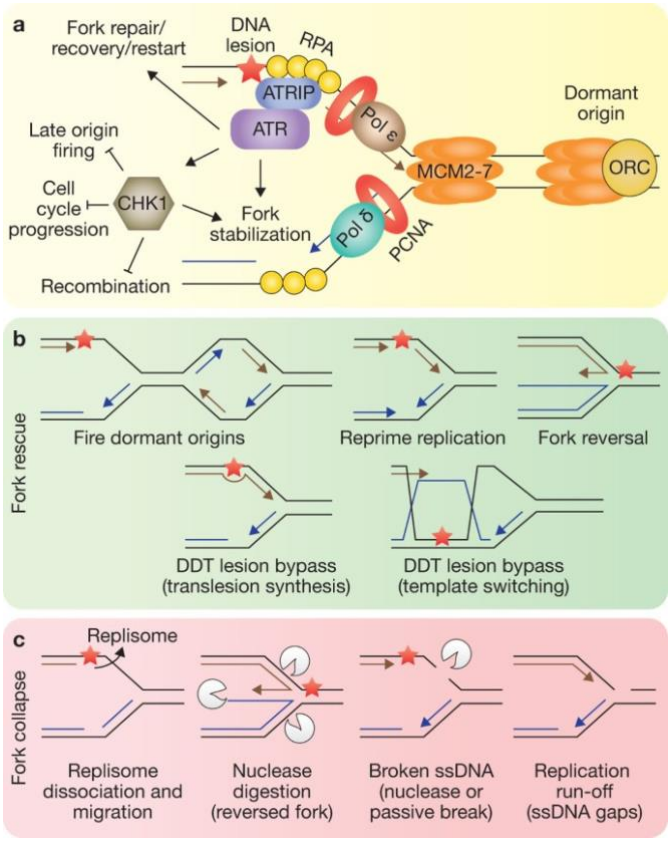


Figure 10: The proteins involved in DNA stress. The red star is where the damage has occurred. **A** Showing some of the proteins involved during DNA stress including RPA and ATR. **B** showing the variations of fork rescue including repriming, fork reversal or DTT pathways. Which are then stabilised by the ATR pathway. **C** shows the possibilities of what could occur during a fork collapse event including replisome migration or replication run off (Zeman and Cimprich, 2014)

1.4. p97's role in Replisome disassembly

p97 is a very important protein in the disassembly of the replication machinery. Once the helicase has been polyubiquitinated on its minichromosome maintenance complex component 7 (Mcm7) subunit, p97 comes along with its major (Ufd1 and Npl4) and minor (Faf1 and Ubxn7) cofactors and binds. The ubiquitin chain is then pulled through the core of p97 first and then the Mcm7 follows, via the mechanism previously mentioned in 1.2.2.2.. Dissociation of the Mcm7 subunit causes the rest of the CMG complex to fall apart and dissociate from the chromatin (Fujisawa et al., 2022, Pye et al., 2007, Ramadan et al., 2017, Tarcan et al., 2022, Villa et al., 2021).

Replisomes are normally dissembled in S-phase, but if this fails, they can be removed from chromatin in mitosis phase (M-phase) of the cell cycle in a backup pathway. However, there are some slight differences between these processes. In S-phase the polyubiquitin chain is linked by K48 residues and is added by the E3 enzyme cullin 2 leucine rich repeat protein 1 ($CUL2^{LRR1}$) (Sonneville et al., 2017, Tarcan et al., 2022). However, in M-phase the chain has K6 and K63 links and is added by the E3 enzyme TRAF interacting protein (TRAIP) (Moreno et al., 2019, Tarcan et al., 2022). This suggests that there could be some extra regulation that is involved in p97 and the disassembly of helicase from chromatin, but further research would need to be conducted to confirm this.

1.5. p97 other replication roles

Although p97 has a very important role in removing the helicase from the chromatin at the end of replication, it also has other roles during replication. Cdt1 is essential for placing the Mcm2-7 on the chromatin, thus is essential to starting replication. It then needs to be inactivated to

stop more Mcm's being recruited resulting in re-replication. p97 aids in the Cdt1 chromatin extraction and degradation, thus stops re-replication occurring (Franz et al., 2011, Ramadan et al., 2017). Another replicating related role of p97 is binding with the Ataxin 3 (ATX3) protein to form a p97–ATX3 complex. In normal physiological conditions, this complex helps to degrade the RING finger 8 (RNF8) protein and inhibits its interaction with the chromatin. However, when there is damage to the chromatin from ionising radiation (IR), the p97–ATX3 complex stops degrading RFN8 so it can interact with the DNA and facilitate with its repair (Singh et al., 2019). When the repair of IR damage has been completed, p97 facilitates removal of repair proteins from chromatin, such as MRE11which aids the repair of a DSB (Kilgas et al., 2021b). If p97 is inhibited, the proteins on the chromatin will not unload but will stay on the chromatin in S-phase and go into M-phase. As p97 has many other functions, inhibition could also cause the shortening of the 5' end of the DNA and faulty repairs (Kilgas et al., 2021a, Tarcan et al., 2022).

1.6. p97 modifications

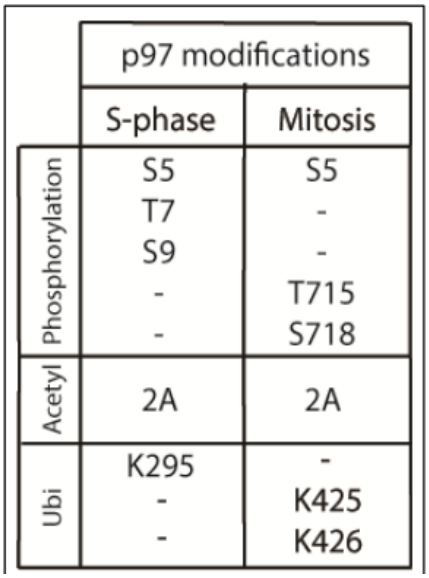
As mentioned previously, during replisome unloading, p97 recognises the polyubiquitin chain on Mcm7, pulling it through its core and unravelling the protein into its primary structure (Bodnar and Rapoport, 2017). However, there are some differences between S and M phases of the cell cycle. During S-phase the ubiquitin chain is added to Mcm7 by $CUL2^{LRR1}$ and is K48 linked while in M-phase it is added by TRAIP and is K6 and K63 linked (Tarcan et al., 2022). For protein degradation, p97 usually recognises K48 linked chains (Lange et al., 2023).

p97 has been found to have about 170 post translational modifications (PTM) sites which include phosphorylation, ubiquitylation, acetylation, SUMOylation and palmitoylation. These

can cause conformational changes impacting its ability to bind to its co factors or substrates. Phosphorylation is reversible and there are currently 2 phosphorylation sites that have been discovered within p97 in proteome-wide experiments. An example is that when there is a DSB, the S784 amino acid is phosphorylated resulting in p97 binding tighter to chromatin, thus causing it to carry out its function. Many ubiquitylation sites have also been identified on p97, however, there has not been many studies focusing on these types of PTMs. It has been suggested that K8 could block substrates from binding. One PTM could also trigger another occurring, such as phosphorylation-dependent ubiquitylation, which is known as crosstalk. These can either have a positive effect on each other (causing the other to bind) or a negative effect (competing to bind) (Hänzelmann and Schindelin, 2017).

p97 is an abundant protein in the cell, it helps to regulate many different processes in the cell with the aid of its 40 cofactors (Buchberger et al., 2015, Yamanaka et al., 2012). However, it is not understood how p97 is regulated to recognise a particular substrate at an appropriate time or how it can bind to a specific adaptor at the appropriate time. PTMs are a good way to regulate such interactions and p97 has many, but their functions are poorly understood, thus there is a gap in the knowledge.

A previous member in the lab carried out a mass spectrometry experiment and identified that p97 has PTM's that are different in S and M phases which are shown in **Figure 11**. It is unknown whether these modifications will impact p97's function or if they will interfere with the binding of its cofactors.



	p97 modifications	
	S-phase	Mitosis
Phosphorylation	S5	S5
	T7	-
	S9	-
	-	T715
	-	S718
Acetyl	2A	2A
Ubi	K295	-
	-	K425
	-	K426

Figure 11: The PTM's of p97 in S and M phases, phosphorylation, acetylation and ubiquitylation. S is serine, T is threonine, A is alanine and K is lysine

2. Aim

Taking the current knowledge about p97 and its PTMs into account, the aim of this project will be to see if the different p97 PTMs in S- phase and M-phase of the cell cycle have an impact on p97's function and how it interacts with certain cofactors. Other proteins that p97 could interact with will also be investigated to identify new potential substrates of p97 during DNA replication.

This will be investigated using the *Xenopus laevis* egg extract system. This is a cell free system that is used to study DNA replication as the egg extract contains a high concentration of the proteins required for DNA replication. It also contains the same proteins that would be present *in vivo*, thus replication occurs like it would in the cell. Egg extract is arrested in metaphase II (met II) of meiosis and can be activated into interphase by the addition of calcium ions along with DNA, making it a good process to analyse DNA replication (Gillespie et al., 2012).
The mutants will include making phospho-mimicking mutants where the amino acid is changed to one that will have the same negative charge as a phosphate group (Schneider and Kabelacˇ, 1998). Secondly, a phospho-dead mutant which has a neutral alanine acid which stops it from being phosphorylated. Finally, ubiquitylation-dead mutants where a lysine amino acid is removed and replaced with another amino acid so a ubiquitin molecule cannot bind (Mattiroli and Sixma, 2014).

3. Materials and Methods

3.1. Tables

Table 1: Table of buffers for p97 purification

Lysis buffer
150 mM Sodium phosphate (Na_3PO_4) pH 7.4 300 mM Sodium chloride (NaCl) 2 mM Magnesium Chloride ($MgCl_2$) 5 mM β- mercaptoethanol (or 2-mercaptoethanol)
Wash buffer
Lysis buffer 60 mM imidazole 0.1 mM PMSF 1 µg/ml of each aprotinin, leupeptin and pepstatin
Elution buffer 1 (E1)- Elution 250 mM
5 mM HEPES 100 mM potassium chloride (KCl) 1 mM $MgCl_2$ 150 mM sucrose 1 mM DTT 0.1 mM PMSF 1 µg/ml of each aprotinin, leupeptin and pepstatin 250 mM imidazole
Elution buffer 2 (E2)- Elution 500 mM
Same as E1 but with 500 mM imidazole
LFB1/50
40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 8 with Potassium hydroxide (KOH) 20 mM K_3PO_4 pH 8 2 mM $MgCl_2$ 1 mM EGTA 2 mM DTT 10% sucrose 50 mM KCl

Table 2: Table of buffers for WRN purification

Lysis buffer
50 mM tris pH7.4 100 mM NaCl 10% sucrose
Wash buffer
Lysis buffer 10 mM imidazole 0.1 mM PMSF 1 μg/ml of each aprotinin, leupeptin and pepstatin
Elution buffer 1 (E1)- His A
50 mM Tris pH 7.4 500 mM NaCl 0.1 mM PMSF 250 mM imidazole
Elution buffer 2 (E2)
Same as E1 but with 500 mM imidazole
Coupling buffer
0.1 M sodium bicarbonate ($NaHCO_3$) 0.5 M NaCl pH 8.3

Table 3: Table of solutions

MMR (10x)
1 M NaCl 20 mM KCl 10 mM $MgCl_2$ 20 mM Calcium chloride ($CaCl_2$) 1 mM EDTA 50 mM HEPES pH 7.8 with Sodium hydroxide (NaOH)
Cysteine
2.2% cysteine 5 mM EGTA pH7.6 with KOH
UEB
50 mM KCl 50 mM HEPES 5 mM $MgCl_2$ 5 mM EGTA pH to 7.6 with KOH
Energy Regenerator (ER)
600 µg/ml Creatine Phosphokinase (Roche), 1 M phosphocreatine (Roche) 10 mM HEPES KOH 7.6
Proteinase K
10 mM Tris-HCl pH 7.5 20 mg/ml proteinase K 50% v:v glycerol
Stop C
5 mM EGTA 0.5% SDS 20 mM Tris-HCl pH7.5
10% TCA
10% w:v TCA 2% w:v Sodium pyrophosphate ($Na_4P_2O_7$) x 10 Water (H_2O)
5% TCA
5% w:v TCA 0.5% w:v $Na_4P_2O_7$ x 10 H_2O
2 x ANIB100
100 mM HEPES pH7.8 100 mM Potassium acetate (KoAc) 20 mM Magnesium acetate (MgOAc) 5 mM Mg-ATP (stock: stock of: 250 mM ATP; 250 mM MgCl2; pH 6.7 with NaOH) 50 mM β-Galactosidase 1 mM spermidine 0.3 mM spermine

2 µg/ml of each aprotinin, leupeptin and pepstatin
1x ANIB100
1 volume of 2x ANIB100 1 volume of H_2O 0.1% Triton X-100 0.1 mM PMSF 500 mM chloroacetamide
LFB1/50 (2)
40 mM HEPES pH 8 with KOH 20 mM Potassium phosphate (K_3PO_4) pH 8 2 mM $MgCl_2$ 10% sucrose 50 mM KCl
LFB1/200
Same as LFB1/50 (2) but with 200 mM KCl
TBST
1x Tris-buffered saline (TBS) 0.1% Tween 20
PBST
1x Phosphate-Buffered Saline (PBS) 0.1% Tween 20

Table 4: Table of recombinant proteins

Recombinant protein
His-X. laevis p97Wt
His-X. laevis p97D1D2
His-X. laevis p97Pmm
His-X. laevis p97Pms
His-X. laevis p97Pdm
His-X. laevis p97Pds
His-X. laevis p97Ubs

Table 5: Table of inhibitors

Inhibitor	**Preparation**
NMS873 (Cayman Chemical Company)	Dissolved in DMSO at 10 mM concentration

Table 6: Table of antibodies

Antibody	**Dilution, buffer, species raised in**	**Provider**
α-Mcm7	1: 2,000, TBST, Sheep	Previously described by (Prokhorova and Blow, 2000)
α-Cdc45	1: 5,00, TBST, Sheep	Previously described by (Gambus et al., 2011)
α-PCNA	1: 2,000, TBST, Mouse	Sigma
α-Psf2	1: 5,00, TBST, Sheep	Previously described by (Gambus et al., 2011)
α-His	1: 1,000, TBST, Mouse	Sigma
α-NPL4	1: 1,000, TBST, Rabbit	Kindly given by Prof Stemmam (Heubes and Stemmann, 2007)
α-p47	1: 1,000, TBST, Rabbit	Kindly given by Prof Olaf Stenman
α-Ubxn7	1: 1,000, TBST, Sheep	Previously described by (Tarcan et al., 2022)
α-Ufd1	1: 1,000, TBST, Rabbit	Kindly given by Prof Stemmam (Heubes and Stemmann, 2007)
RPA (sera)	1: 1,000, PBS, Rabbit	Raised in the lab
WRN (sera)	1: 1,000, TBST, Rabbit	Raised in the lab
HRP-sheep-IgG	1: 10,000, TBST	Sigma
HRP-mouse-IgG	1: 5,000, TBST	Sigma
HRP-rabbit-IgG	1: 25,000, TBST	Sigma

3.2. Plasmid work

3.2.1. Site directed mutagenesis (SDM)

A 25 µl SDM reaction was set up containing 1x Q5 reaction buffer (New England BioLabs), 200 µM Deoxynucleoside triphosphates (dNTP's) (New England BioLabs), 0.5 µM of the forward and reverse primer, 1 ng/µl of the plasmid, 0.02 U/µl of Q5 High- Fidelity DNA polymerase (New England BioLabs) and 1x Q5 High GC Enhancer. These were added to a Polymerase chain reaction (PCR) tube and topped up with nuclease free water (to 25 µl). These were then run on a PCR machine with the following annealing temperatures: 50°C,

50.6°C, 51.9°C, 53.5°C, 56°C, 59.2°C, 62.9°C, 66°C, 68.5°C, 70.2°C, 71.5°C and 72°C. The following parameters were used: initial denaturation 98°C for 30 seconds (s), then 25 cycles of: denaturation 98°C for 10 s; annealing of temperatures outlined previously for 30 s, extension 72°C for 1 minute (min) and a final extension 72°C for 2 mins. The samples were then held at 4°C until they were taken off the machine.

3.2.2. Agarose gel

A 1 L mix of 1X Tris-borate-EDTA (TBE) was prepared, and 0.8% agarose was added and then boiled. This was cooled slightly and a 1/10,000 dilution of SYBR safe gel stain (Introvegen) was added and set in a cassette. The tank was filled with 1X TBE. Blue/orange DNA dye (Promega) was added to the samples and the 1 Kb Plus DNA ladder (Introvegen). This was run for 1 hour (h) at 100 V and visualised under UV light.

3.2.3. Miniprep

Precultures of NEB-10 β bacteria (4 x 10 ml) containing the newly synthesised p97 mutant plasmid (as described later in 3.3.2.) were made and the method of the QIAprep Spin Miniprep Kit (Qiagen) was followed. To summarise, the bacteria with the plasmid was pelleted and resuspended in 250 µl of P1 buffer. Then 250 µl of the P2 buffer was added and mixed. Then 350 µl of N3 buffer was added, mixed and centrifuged for 10 mins at 18°C at 17,900 x g. The supernatant was transferred to a QIAprep spin column (Qiagen) and centrifuged for 1 min. Then 750 µl of PB buffer was added to a column and centrifuged as outlined in last step. The column was centrifuged again and placed into a new microcentrifuge tube. Next, 50 µl of the EB buffer was added and was centrifuged again. The

flow through was then placed back into the column and spun again for 1 min. The flow through was collected (plasmid sample) and a DNA concentration test was carried out as outlined below in 3.2.4. Method can be accessed at: (QIAGEN, 2010).

3.2.4. Plasmid DNA concentration

The NanoDrop™ 2000 (ThermoFisher) was calibrated using the EB buffer (Qiagen) and 1 µl of the plasmid sample was read to give the ng/ µl of the sample. The A260/A280 ratio was noted. The samples were then sent off to plasmidosaurus for sequencing.

3.3. Bacteria Work

3.3.1. Transformation

The plasmid with a concentration of ~115 ng/µl was taken (1 µl) and added to 20 µl bacterial cells (Rosetta or BL21) and incubated in ice for 30 mins. They were then heat shocked at 42°C for 30 s and then placed on ice for 5 mins. Then 300 µl of Super optimal broth with catabolite repression (SOC) outgrowth medium (New England BioLabs) was added and incubated at 37°C for 1 h in a shaking incubator at 200 revolutions per minute (RPM). Next, 120 µl of the bacterial culture was plated on an agar plate with antibiotics (as described below) and incubated at 37°C overnight.

Plasmids: pQE80 p97 (and its mutants, outlined in **Table 7**) and PET28a WRN

Bacteria cells: p97 in Rosetta (DE3) pLysS Competent Cells (Novagen) and WRN in BL21-(DE3) Competent Cells (Fisher Scientific), to extract the plasmid, NEB-10-β Competent cells (New England Bio labs)

Antibiotics: 0.1 mg/ml Ampicillin for p97 and 0.05 mg/ml Kanamycin for WRN.

3.3.2. Growing Bacteria to Express Proteins

For 1 L: One colony (or tip of cells from a glycerol stock) was added to 10 ml Luria Bertani broth (LB) with antibiotics (as described above in 3.3.1) and incubated at 37°C overnight in a shaking incubator at 200 RPM. The pre-culture was added to 1L of LB (with corresponding antibiotic) and placed in a shaking incubator (200 RPM) at 37°C. This was done until the optical density (OD) reached 0.4 to 0.5 at a wavelength of 595 nm (measured by an P300 NanoPhotometer, Implan) and 1 mM of Isopropyl β- d-1-thiogalactopyranoside (IPTG) was added and left for another 3 h. The cultures were then added to 1 L centrifuge bottles and centrifuged at 6,000 x g for 15 mins at 4°C. The supernatant was discarded, and the pellet was resuspended in 30 µl of lysis buffer (outlined in **Tables 1** and **2**) and stored at - 18°C.

3.3.3. p97 Purification

The 4 L resuspended bacterial pellet was defrosted and supplemented with the following: 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM imidazole, 1 mg/ml lysozyme, 25 U/ml of benzonase nuclease (SIGMA) and 2 cOmplete, Ethylenediamine tetraacetic acid (EDTA) protease inhibitor cocktail tablets (Sigma-Aldrich). This was mixed for 30 mins at room temperature (RT) and then Sonicated (Qsonica 700) 10 times for 5 s at a 50% duty cycle. The lysate was transferred to 50 ml Nalgene centrifuge tubes and centrifuged at 14,000 x g, at 4°C for 30 mins. A 40 µl sample of supernatant (input) was taken. The remaining was added to 4 ml prewashed His Pur Ni-NTA (Nickel-nitrilotriacetic acid) Resin beads (ThermoFisher) and mixed at 4°C for a minimum of 2 h.

The tubes were then spun for 1 min at 4°C at 1,000 x g, and 40 µl of supernatant (depleted) was taken. The rest of the supernatant was removed. Then 5 ml of wash buffer was added and the beads were transferred to a 10 ml Poly-Prep Chromatography Column (Bio-Rad). This was then pulse centrifuged and 40 µl of the flow through was collected. The rest of the flow through was discarded and the process repeated 2 more times. Next, 1 ml of E1 was added (if using) or E2 was added, and pulse centrifuged until a 1 ml fraction was collected in a 1.5 ml Eppendorf tube. If using E1 and E2, this was repeated with E1 5 times and E2 five times. If only using E2, this was only repeated 5 times. The buffers used are outlined in **Table 1**. With a pipette tip, some of the beads were removed that were left in the column. To a sample of 10 µl for each elution, 10 µl of 2x NuPAGE SDS loading buffer was added. For the input, depleted, washes and beads 40 µl was added. The samples were boiled at 95°C for 5 mins. The samples were run on a NuPAGE- SDS gel as described in 3.7.1.. The fractions, if required were then dialysed and the concentrations were also analysed via a Bradford assay and Bovine Serum Albumin (BSA) gel (outlined in 3.7.1. – 3.7.4.).

3.3.4. WRN purification

A 2 L pellet were defrosted and brought to 500 mM NaCl. Then was supplemented with 0.1 mM PMSF, 5 mM imidazole, 1 mg/ml lysozyme, 25 U/ml of benzonase nuclease (SIGMA), 2 cOmplete, EDTA protease inhibitor cocktail tablets (Sigma-Aldrich) and 0.1% Triton 100x. The purification was then the same as the p97 purification (3.3.3.) but with the buffers outlined in **Table 2**.

3.4. Working with *X. Laevis* egg extract

3.4.1. Preparing unactivated *X. Laevis* egg extract

The protocol for the preparation of *X. Laevis* egg extract is outlined in the (Gillespie et al., 2012) paper.

Around 2- 7 days prior to egg collection, 10 female *X. Laevis* frogs were injected with 150 U of a follicle stimulating hormone, Foligon (Intervet). The day before collection, 500 units of serum gonadotropin, Chorulon (Intervet) was injected and frogs were kept in individual tanks overnight at 37°C containing 1 L of 1x Marc's modified ringer's buffer (MMR). Good eggs were identified and collected in a 1 L beaker. Characteristics to look for are darker colour animal pole, than the light colour vegetable pole and not white or joined together.

Eggs were rinsed with 1x MMR and as much liquid as possible was removed. Cysteine was added to de-jelly the eggs in three repeats (maximum 10 mins). Eggs were rinsed with 1x MMR and then with UEB plus 2 mM Dithiothreitol (DTT). To remove any swollen white eggs a Pasteur pipette was used. Then 1 ml of UEB (added with 2 mM DTT, 10 μg/ml each of aprotinin, pepstatin and leupeptin (Lifesciences) and 50 μg/ml cytochalasin D, Sigma) was added to Ultra Clear 18 x 95 mm centrifuge tubes (Greiner Bio-One) and topped up with eggs. This was then centrifuged in a swinging bucket JS13.1 rotor (Optima) at RT, 3,000 RPM for 1 min. Any white apoptotic eggs collecting at the top of the tube were removed.

The compacted eggs were then centrifuged again in a swinging bucket JS13.1 rotor (optima) at 12,000 RPM, RT for 10 mins. From now on, the eggs were kept on ice. The brownish layer (cytoplasmic fraction) was collected with a 1 ml syringe and a 20-G needle (and the yellow lipid plug and grey insoluble palette was discarded). The extract was placed in a falcon tube

and supplemented with 10 µg/ml cytochalasin D and 15% LFB1/50. This was then transferred into SW50 ultracentrifuge tubes (Beckman Coulter) and centrifuged in a SW55 rotor (Beckman) at 30,000 RPM, 4 °C for 20 mins.

The lipid plug (yellow layer) was removed, and the cytoplasmic layer (golden) was collected. If the mitochondria layer (the grey at the bottom of cytoplasm) was disturbed, the tube was discarded and the next one was utilised. After, 100% glycerol was added to a 1% concentration and egg extract frozen in 20 µl drops (using liquid nitrogen) and stored at at 80°C.

3.4.2. DNA synthesis experiments

3.4.2.1. Activation

As the egg extract is halted in metII of mitosis, it needs to be activated to complete replication. Adding $CaCl_2$ at a 0.3 mM concentration mimics the natural increase of calcium (Ca^{2+}) after fertilisation, triggering the extract to go into interphase. DNA replication needs a lot of energy which can be added to the extract in the form of the ER solution at a 1/40 dilution. In order to block the cell entering other stages of the cell cycle, protein synthesis (mainly cyclin synthesis) needs to be blocked, which can be done by the addition of 250 µg/ml of cycloheximide (CHX) (Sigma) (Gillespie et al., 2012).

3.4.2.2. TCA DNA synthesis assay

To check the extracts replication potential (with any required modifications) a TCA replication assay can be carried out which is described in the paper by (Gillespie et al., 2012).

The egg extract was activated with 0.3 mM of $CaCl_2$, 250 µg/ml of CHX and 1/40 ER for and incubated at RT 15 mins. After the initial 15 mins, 10 ng/µl of de-membraned sperm nuclei and 16.5 nM α-[p32]dATP (Perkin Elmer) was added. At this point, any buffers and proteins could be added (at their required concentrations, mentioned in results) and separated into 5 µl aliquots. The samples were incubated at 23°C until the respective time points had been reached. At each time point, replication was stopped by the addition of 160 µl Stop C (supplemented with 0.2 mg/ml proteinase K, Macherey Nagel) and incubated at 37°C for a minimum of 30 mins (to degrade all proteins).

Samples were then transferred to 5 ml tubes with 4 ml of 10% (Trichloroacetic acid) TCA (to precipitate the DNA) and incubated at 4°C for a minimum of 1 h. Then 40 µl of each sample was taken and placed on a 25 mm Whatmann paper filter (GE healthcare life sciences), which was needed for the total radiation level. The rest of the sample was poured through a 25 mm glass fibre filter (Fisherbrand) and placed on a manifold with a vacuum (Millipore). The tubes were then washed with 10% TCA. The filters were washed with 5% TCA and rinsed with 70% ethanol. All filters (paper and glass fibre) were left to dry under a lamp. Papers were placed into scintillation counter tubes and 0.5 ml Ultima Gold F scintillant (Perkin Elmer) was added. The tubes were then placed into a scintillation counter for quantification of the radiation levels.

To calculate the total DNA synthesis in ng/µl, the p32 on the glass fibre filter was divided by the amount on the paper filter. To convert this into ng DNA/ µl of extract, this was multiplied by 0.654 (assumes all dNMTP's are incorporated equally and all have a weight of 327 Da) (Gillespie et al., 2012).

3.4.2.2.1 Testing buffers and p97 mutant proteins via TCA

Method was the same as outlined in the TCA DNA synthesis assay (3.4.2.2.) but with the addition (after the initial incubation) of LFB1/50, E1, E2, Wt p97 or the p97 mutants (outlined in **Tables 1** and **7**) at varying concentrations and volumes depending on the experiment (outlined in results).

3.4.2.3. Chromatin isolation time course

To check the different protein interactions during DNA replication under different conditions, a chromatin isolation time course was carried out which is described in the (Gillespie et al., 2012) paper. For a general chromatin isolation, the egg extract was activated with $CaCl_2$ at a 0.3 mM concentration, 250 µg/ml of CHX and 1/40 of ER and incubated for 15 mins at RT. A sample of 20 µl was taken for each condition as a chromatin specific control (-DNA). After 15 mins, 10 ng/µl of de-membraned sperm nuclei was added and incubated for a further 15 mins at 23°C. After incubation, any proteins, buffers and inhibitors can be added at this point at their required concentrations, outlined in results. The samples were then split into 20 µl aliquots in Treff tubes (TreffLab) and incubated at 23°C until the time point was reached.

At the time points, the reaction was stopped by increasing the extract volume by diluting it with the addition of 500 µl of ice cold 1x Acetate Nuclear Isolation Buffer (ANIB100). This was then underlaid with 100 µl of ANIB100 (supplemented with 20% sucrose) creating a sucrose cushion. This was then centrifuged in a S417R swing bucket rotor (Eppendorf) for 5 mins at 2500 RPM at 4°C. The ANIB100 buffer was removed, and the tube was washed twice with 100 µl of ANIB100 (to reduce contamination). Most of the sucrose cushion was removed (approximately 70 µl) and the rest was spun in a S41802 fixed angle rotor

(Eppendorf) at 18°C, maximum speed for 2 mins, ensuring that the hinge of the tube was facing outwards to indicate the side of the tube where the chromatin pellet collects. The remaining liquid was removed, and the pellet was resuspended in 30 µl 2x NuPAGE SDS loading buffer. The same process was carried out for the –DNA samples (negative control). A 5 µl sample of the extract was taken and added to 45 µl of 1x NuPAGE-SDS loading buffer. All samples were boiled at 95°C for 5 mins and ran on a NuPAGE-SDS gel (as described in 3.7.1).

3.4.2.3.1. Testing mutant p97 proteins via chromatin isolation

Method was the same as outlined in the chromatin isolation time course (3.4.2.3.) but with the addition (after the second incubation) of either LFB1/50 or E1 as the control, and Wt p97 or the p97 mutants (outlined in **Tables 1** and **7**) at a final concentration ranging from 0.5 mg/ml to 0.3 mg/ml depending on the experiment (outlined in results).

3.4.2.3.2. Effect of a p97 inhibitor via chromatin isolation

To see if various proteins behave differently when p97 activity is blocked during replication, a p97 inhibitor was added. The chromatin isolation was performed the same as outlined in the chromatin isolation time course (3.4.2.3.) but with the addition of Dimethyl sulfoxide (DMSO) as the control and a final concentration of 50 µM of NMS873 (p97i) in the test sample after the second incubation.

3.4.2.4. His- pulldown for protein and cofactor interactions

The egg extract was activated as described above in 3.4.2.1. and the extract samples were split into 4 x 30 µl aliquots and E1, Wt p97 and two of the p97 mutants were added at a final concentration of 0.27 µg/µl to the extract. These were then incubated for 30 mins at 23°C. Then 130 µl of LFB1/50 (2) was added into each tube and a 30 µl sample (extract) taken from each tube. The tubes were then centrifuged at max speed in a fixed angled S417R swing bucket rotor (Eppendorf) at 4°C for 10 mins. An input sample (30 µl of the supernatant) was taken, and the rest was added to 30 µl prewashed Dynabeads His-Tag Isolation and Pulldown (Invitrogen) and rotated at 4°C for 2 h. The tubes were then centrifuged for 1 s and placed on a magnetic rack. A 30 µl sample of the supernatant (Depleted) was taken and the rest was discarded. The beads were then washed and mixed for 5 mins on 3 different occasions with 1 ml of the following ice-cold buffers: LFB1/50 (2), LFB1/50 (2) with 0.1% Triton 100x and LFB1/200 with 20 mM imidazole. The samples were then centrifuged for 1 s and 30 µl 2x NuPAGE SDS loading buffer added and vortexed. To the extract, input and depleted samples, 10 µl 4x NuPAGE SDS loading buffer was added. All samples were then boiled for 5 mins at 95°C. The beads were then vortexed and placed on a magnetic rack. The supernatant was then transferred to fresh tubes (pulldown samples). The samples were then run on a NuPAGE- SDS gel (as described in 3.7.1.).

3.4.2.5. Immunoprecipitation to test sera

For each experiment, 100 µl of egg extract was activated as above in 3.4.2.1.. Then 400 µl of LFB1/50 was added and 20 µl of the sample was taken, for the extract sample. This was then centrifuged in a fixed angled S417R swing bucket rotor (Eppendorf) at max speed for 15 mins at 4°C. The supernatant was then split into four 100 µl aliquots. Then 20 µl of the

remaining supernatant was taken for the Input samples. To each of the extracts aliquots, 5 µl of the pre-sera and sera from both rabbits were added and incubated on ice for 1 h. Samples were centrifuged for 1 s and added to pre-washed Protein A Dynabeads (Invitrogen) for immunoprecipitation and rotated at 4°C for 1 h. Samples were then spun for 1 s and placed on a magnetic rack. A 20 µl sample of the supernatant was taken (Depleted) and the rest was discarded. The beads were then washed and mixed for 5 mins on 3 occasions with 1 ml of the following ice-cold buffers: LFB1/50, LFB1/50 with 0.1% Triton 100x and LFB1/50. The samples were centrifuged for 1 s and 30 µl of 2x NuPAGE SDS loading buffer was added and they were vortexed. To the extract, input and depleted samples, 20 µl of 2x NuPAGE SDS loading buffer was added. All samples were then boiled for 5 mins at 95°C. The beads were then vortexed and placed on a magnetic rack, the supernatant was then transferred to fresh tubes (IP samples). Samples then ran on a NuPAGE- SDS gel (as described in 3.7.1.).

3.5. Statistics

The bands in the his-pulldown samples were quantified using ImageJ using the uncalibrated OD setting. Each of the mutant protein's bands were normalised by dividing the value from the specific protein by the band in the his-blot (immunoprecipitation efficiency). The fold change was then calculated by dividing the mutant protein normalised value by the Wt's value. A two-sample student's T-test assuming equal varices was then carried out at a 95% significance level. This was done using five repeats for each protein.

3.6. Potential protein interactions

To find potential protein candidates that could interact with p97, a previous member of the labs p97 immunoprecipitation (IP), His-Ubi pulldown (with p97i) and p97i chromatin mass spectrometry (chromass) results were analysed as described below.

3.6.1. Analysis

Analysis of the results (IP, His-pulldown and p97i chromass) was conducted using the software program called Scaffold. The proteins were sorted from the highest to the lowest number of peptides. The cut-off point was set at 10 peptides long. The number of peptides in the control was noted and the total enrichment was calculated (test number of peptides divided by the controls number of peptides), with the cut off point for the total enrichment being 2 times. Any proteins that were identified to be from contamination, such as keratin, were omitted from the results. Then the same was repeated for the proteins with a 95% + identity of Glycine- Glycine (Gly-Gly) residues to see if there was any residues which were ubiquitinated. The peptide number of the ubiquitin sites were also calculated and recorded. This was done for the IP and his-pulldown in S-phase and the IP and p97i chromass experiment in M-phase separately.

Once the two sets of data had been collected for S-phase, they were then merged. This only contained the proteins which were identified by the mass spectrometry in both the IP and His-pulldown in the final table. The order that it is presented in is the proteins having the highest number of peptides to the lowest from the IP results. This was then repeated for the results from the M-phase IP and p97i chromass data. This gave 3 tables, for S phase, the identified

proteins (**Table 9**) and the proteins with Gly-Gly residues (**Table 10**). For M-phase just the proteins identified, **Table 11.**

3.7. General methods

3.7.1. NuPAGE-SDS gel

PageRuler Plus Prestained protein Ladder 10 to 250 kDa (ThermoFisher) (5 µl) was loaded onto a precast NuPAGE 4-12% gradient Midi Bis-Tris gels (Invitrogen).

For gels for protein purification: 10 µl input, depleted, washes, beads and 5 µl elution's were loaded.

For BSA gel samples: 10 µl BSA and protein samples were loaded.

For gels with chromatin isolations: 5 µl extract, 14.5 µl – DNA and, 5 µl time points were loaded.

For His-pulldown samples: 10 µl of each sample were loaded.

For IP samples: 5 µl of each sample were loaded.

For the protein concentration gel (pure protein not BSA diluted proteins): 5 µl each sample loaded.

The gels were then run in a XCell4 Sure Lock (Invitrogen) tank at 160 volts (V) for 1 h with 1x 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (Novex).

For the elution and BSA gels, they were stained for 30 mins in Coomassie Stain (1 g Coomassie Brilliant Blue R-250, 400 ml methanol, 100ml acetic acid, 500ml H_2O). Then washed several times with distaining solution (400 ml methanol, 100 ml acetic acid, 500 ml H_2O), until the bands on the gel were distinguishable. Gels were then placed in an acetate pouch and scanned.

3.7.2. BSA concentration sample preparation

A 10 mg/ml solution of BSA was serially diluted into 10 µg/µl, 5 µg/µl, 2.5 µg/µl and 1.25 µg/µl with either elution 250 mM, elution 500 mM, LFB1/50 or coupling buffer (depending on the protein being tested, described in results). These were then mixed with equal quantities of 2x NuPAGE SDS loading buffer.

The protein that was being tested was made into a 2 µl sample (mixed with 2x NuPAGE SDS loading buffer). This was then serially diluted to make 1 µl and 0.5 µl and the addition of 1x NuPAGE SDS loading buffer. These were then run on a NuPAGE- SDS gel (as described above, 3.7.1.).

3.7.3. Bradford assay

A standard curve was made by using 1 mg/ml of BSA and adding this to H_2O and Coomassie Plus (Bradford) Assay Reagent (Fisher Brand) into samples ranging from 2 mg/ml to 12 mg/ml. These were then incubated for 5 mins in the dark and three repeats of the OD were taken at a 595 nm wavelength.

Protein samples of 1x and 4x were prepared, incubated and the OD was measured the same as the BSA samples. The concentration was calculated using the standard curve.

3.7.4. Dialysis

The required size of Pur-A-Lyzer (Sigma-Aldrich) dialysis column (at least 3x smaller than the size of the protein) was activated and the fraction with the most amount of p97 or WRN

(or if too concentrated, diluted slightly) was added. This was placed in 1 L of the required buffer (LFB1/50 or coupling buffer depending on the protein, described in results) and mixed at 4°C overnight. The protein was transferred to a 1.5 ml Eppendorf tube and the concentration was tested via a BSA gel (outlined above, 3.7.2.) and frozen in -80°C.

3.7.5. Western blot

Samples were run as outlined above on a NuPAGE-SDS gel (3.7.1.). If needing to see the histones, after the gel had run, the bottom of the gel (histones) was cut off, washed 3 times with water and stained with SimplyBlueTM SafeStain (Invitrogen) overnight. This was then washed 3 times in water before being scanned to obtain a loading control.

The rest of the gel was then transferred to a nitrocellulose membrane (Fisher scientific) (if blotting for RPA) or a Polyvinylidene difluoride (PVDF) membrane (Merck Millipore) for all other proteins via a wet transfer system (Bio Rad), running for 1 h 15 mins at 100 V. The membranes were then blocked in 5% milk in TBST for 1 h. The membranes were then washed 3 times in TBST (and cut if required). They were then left to rotate at 4°C overnight with the required primary antibody (either in 5% milk, for RPA and Psf2 or 3% BSA for all other antibodies. All in TBST apart from RPA which was in PBS). All primary antibodies were supplemented with 0.02% Sodium azide (NaAzide).

The membranes were then washed 3 times in TBST and then rotated for 2 h at room temperature in the corresponding secondary antibody, in 5% milk with TBST. The secondary antibodies that were used were: Anti-sheep IgG from donkey, conjugated to horseradish peroxidase (HRP) (Sigma- Aldrich), Anti-mouse IgG from goat conjugated to peroxidase

(Sigma-Aldrich) and Anti-rabbit IgG (H+L) from goat (Sigma-Aldrich). Membranes were then washed 3 times in TBST, sprayed with Western BrightTM ECL-spray (Advansta) and films developed to show the results.

4. Results

The aim of the project was to see if the PTM's impact p97's function in S-phase and M-phase of the cell cycle and if it impacted p97's interaction with its cofactors. It also investigated other potential proteins that p97 could interact with during DNA replication.

p97 is in a high abundance in *Xenopus* egg extract at 0.25 mg/ml (Heubes and Stemmann, 2007) thus, it cannot be immunodepleted from the egg extract. Therefore, to overcome this factor, a high concentration of recombinant p97 could be added to the extract (about a 0.5 mg/ml final concentration) to override the endogenous p97. Previous studies have added recombinant D1D2 p97 at a final concentration of 0.5 mg/ml in *Xenopus* egg extract and this has not affected the replication but did block unloading of replisomes as expected (Heubes and Stemmann, 2007, Moreno and Gambus, 2020, Priego Moreno et al., 2014, Tarcan et al., 2022)
.

4.1. Making p97 mutants

In order to compare the functions of all of the mutants, they all needed to be expressed and purified. The original p97 gene expression plasmid needed to be mutated via SDM using specially designed primers. Once the plasmid mutations had been produced, these could then be used to express and purify the proteins to be tested for functionality.

4.1.1. Site directed mutagenesis

First, the primers were designed as oligonucleotides and then using PCR, used to make the desired plasmids shown in **Table 7**. For the catalytically dead mutant (D1D2) the glutamine (G) was changed for a glutamic acid (E). For the phosphorylation mutants a phospho-

mimicking mutant, changing the threonine (T) or serine (S) for a Aspartic acid (D). A phospho-dead mutant was also made, changing the T or S for an alanine (A). Finally, for the ubiquitylation dead mutants, the K was changed for an arginine (R). The following mutants were identified that needed to be produced: ATPase (D1D2), Phosphorylation Mimicking M-phase (Pmm), Phosphorylation Mimicking S-phase (Pms), Phosphorylation Dead M-phase (Pdm), Phosphorylation Dead S-phase (Pds), Ubiquitylation dead S-phase (Ubs), Ubiquitylation dead M-phase (Ubm).

In order to get the best chance of the SDM to work, a gradient PCR reaction was set up with each tube having a different temperature. The results were then run on an agarose gel and the PCR product with the strongest band at 7152 base pairs (bp) (size of the plasmid) and with the least amount of incomplete products was then ligated and transformed into bacteria. The bacteria were then amplified to retrieve the mutated plasmids and sent off for sequencing using Plasmidosaurus. The results were then retrieved from the SnapGene program and aligned to the original plasmid via Blast protein alignment. In order for a sequence to be correct, the new sequence must have been the same as the original plasmid apart from the one or two amino acids that were being mutated and have no other random mutations, insertions or deletions. The only plasmid that did not come back as correct was Ubm.

Table 7: The primers created for each of the p97 mutants with the amino acid change stated and the primer sequence shown. No primer was made for D1D2

Mutant	Mutation	Forward primer sequence	Reverse primer sequence
D1D2	Q305E, Q578E		
Wt	E305Q	CTTTATAGACGAGCTG GATGC	ATAATGGCAGGGGC ATTC
	E578Q	CTTCTTTGATGAATTGG ACTCCATCGCTAAG	AGGACACAGGGAGC AGCC
Pmm	T715D, S718D	cccgacGCTATGGAAGTG GAAGAAGAC	gttatcCTGCCTTTCCC GCTCTCT
Pms	T7D, S9D	agacGATGATTTGTCCAC TGCAATC	ttgtcATCTGATCCGGA AGCCAT
Pdm	T715A, S718A	cccgccGCTATGGAAGTG GAAGAAGAC	gttagcCTGCCTTTCCC GCTCTCT
Pds	T7A, S9A	agccGATGATTTGTCCAC TGCAATC	ttggcATCTGATCCGG AAGCCAT
Ubs	K295R	GGAGGCTGAGaggAATG CCCCTG	TCAAAAGCTTTGCG CAGGTTAC
Ubm	K425R, K426R	GGCCATAAGGaggaggAT GGACCTCA	TGGAGAGCAGCTTC TGAG

4.2. Purification of p97

Once the sequence was correct, the proteins could be purified. A stock concentration of purified protein of least 4 to 5 mg/ml was required for experiments in the egg extract. As a high final concentration was needed to outcompete the endogenous p97 protein and the egg extract can only be diluted to a maximum of 1/8 vol to retain efficient replication capabilities (Heubes and Stemmann, 2007).

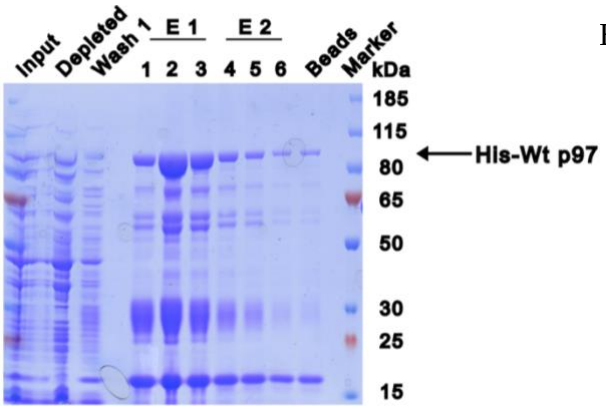
The first step of p97 expression and purification was to test different conditions. For example, for transformation, different cells were trialled (Rosetta and BL21), different volume of cells ranging from 10 µl to 40 µl, the amount of plasmid added ranging from 1 µl to 2 µl and varying

heat shock times ranging from 30 s to 45 s were all trialed. The optimal conditions were found to be 20 µl Rosetta with 1 µl plasmid with 30 s heat shock.

Once the bacteria pellet had been collected and resuspended in the lysis buffer. The purification could then be carried out. This was done using nickel affinity beads as the p97 protein is His-tagged, the histidine (His) binds to the nickel coating in the beads. This was then washed to remove any unbound proteins. The protein was then eluted with the addition of imidazole which competes with the His, causing the protein to be removed from the beads and be eluted (Schafer et al., 2002).

As mentioned in materials and methods (3.), two concentrations of imidazole were tested. Initially 250 mM imidazole was used, however, in most of the purifications, not enough protein was eluted, so 500 mM was later used. All elution's are shown in **Figure 12**. Wt, D1D2 and Pmm (**Figure 12 A-C** respectively) used 250 mM and 500 mM imidazole while Pms, Pdm, Pds and Ubs (**Figure D- G** respectively) just used 500 mM imidazole. Overall, all mutants were successfully purified.

A)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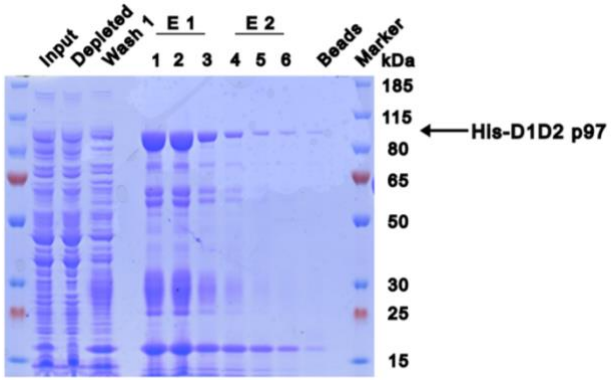
His-Wt p97

B)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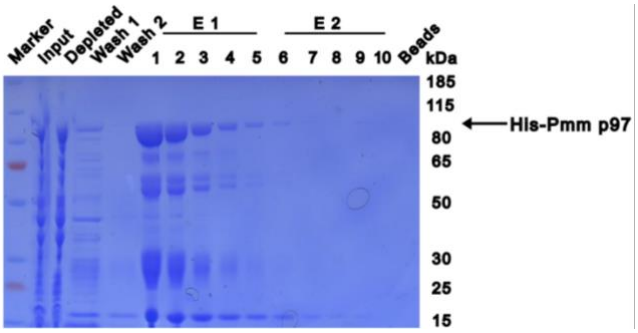
C)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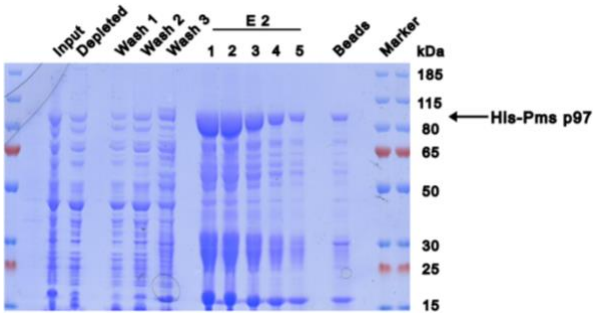
His-Pmm p97

D)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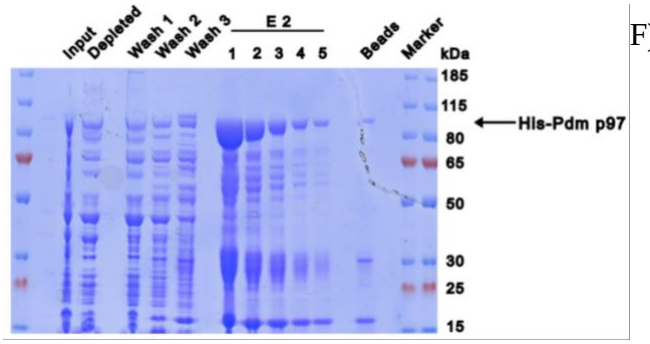
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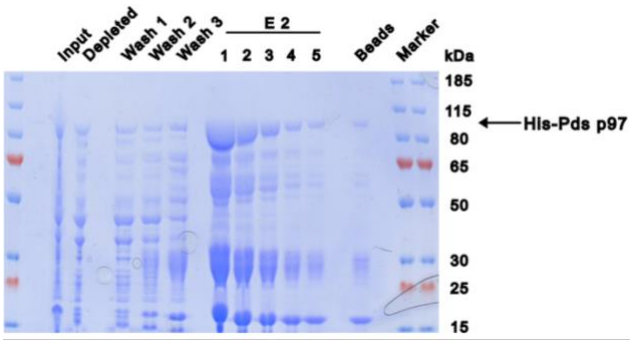
F)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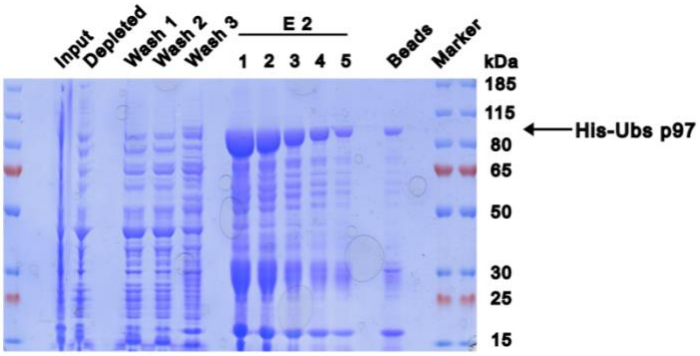
His-Pds p97

G)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His-Ubs p97

Figure 12: The SDS-page gel of the p97 purifications with the input, depleted, washes, elutions and beads shown. E1 is p97 elution buffer 1 with 250 mM imidazole and E2 is p97 elution buffer with 500 mM imidazole. **A** is the Wt, **B** D1D2, **C** Pmm, **D** Pms, **E** Pdm, **F** Pds and **G** Ubs. The input contains the supernatant with the soluble protein after bacterial lysis. Depleted sample contains the proteins supernatant that didn't bind to the Ni-NTA beads after being mixed. The washes contain the unspecific bound proteins to the beads. The elution's contain the proteins that have been eluted with the corresponding elution buffers (all but Wt had the most protein in fractions 1 and 2 (F1 and F2), while the Wt had the most in F2 and F3). Finally, the beads show the proteins that were still bound after all of the elution's have taken place

The concentrations of all proteins were then tested via a BSA gel (**Figure 13**) and a Bradford assay (**Table 8)**. In a BSA gel, the BSA can be diluted to a known concentration which will give different size bands, then the protein with the unknown concentration can be run on the gel and the band size of the unknown can be compared to the BSA to estimate the concentration of the protein of interest and other proteins present in the sample. In a Bradford test, the total concentration of all proteins in the sample is measured using a spectrophotometer. A standard curve of a known protein concentration is made and the unknown protein samples concentration is estimated from the standard curve. The BSA gels (**Figure 13**) showed that the concentrations are all around 4 – 5 mg/ml for all proteins, however, the Bradford (**Table 8**) showed quite different values with the smallest being 8.54 mg/ml (Wt) and the highest being 18.45 mg/ml for Pds. There are quite a few smaller bands present in all of the samples (either from p97 degradation products or contaminants). The BSA comparison is a better method for estimating the concentration of just the full length p97 as it shows the proteins present and their sizes, so can be distinguished between. Whereas the Bradford assay gives one reading for all

proteins present and specific concentrations of proteins cannot be distinguished. Thus, it is not surprising that the Bradford assay showed higher concentrations.

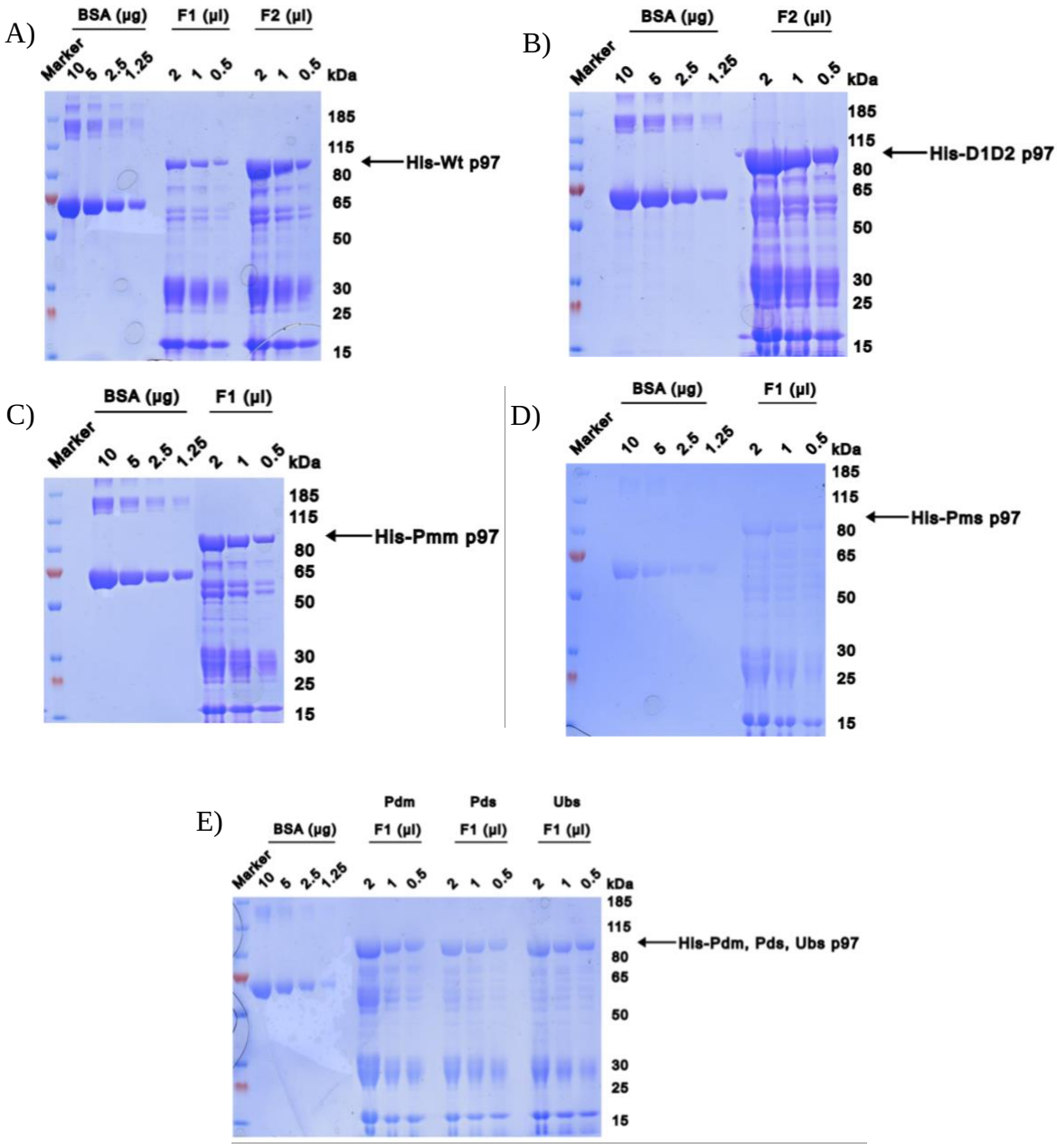

Figure 13: The BSA gels of all the mutant p97 with the fraction stated. **A** with Wt, **B** D1D2, **C** Pmm, **D** Pms, **E** Pdm, Pds and Ubs. D1D2 and Pms. All but Wt and D1D2 were dialysed into LFB1/50 while Wt and D1D2 were left in the elution buffer with 250 mM imidazole

The concentrations from the Bradford assay and BSA tests were both taken into consideration and a final concentration for each protein was concluded in **Table 8**. The BSA test was the basis of the concentration however, the Bradford assay results were used to check that the concentration was roughly 3 or 4 times higher than the BSA results which correlates to the amount of protein present in the sample that were smaller than 97 kDa.

Table 8: The concentrations of all p97 proteins made measured by a Bradford assay in mg/ml, and new estimates were made from the combined concentration when taking BSA and Bradford tests into account

Protein	Bradford assay Concentration (mg/ml)	Combined concentration (mg/ml)
Wt	8.54	3
D1D2	14.38	5
Pmm	11.36	3
Pms	15.66	3
Pdm	14.83	4
Pds	18.45	5
Ubs	12.09	3

As **Figures 12** and **13**, show that there is quite a lot of other smaller bands present that are not at 97 kDa (p97's size). To see what they are, a western blot was carried out to see if they are his-tagged (smaller fragments of p97) or if other contaminating proteins have also been purified. Although it is not very clear, **Figure 14** shows multiple His-tagged bands present, not just one at 97 kDa in all samples. This suggests that there could have been a lot of truncated p97 protein being purified as well as the correct sized protein. This would increase the Bradford assay measure of concentration, however, it would be non-functioning.

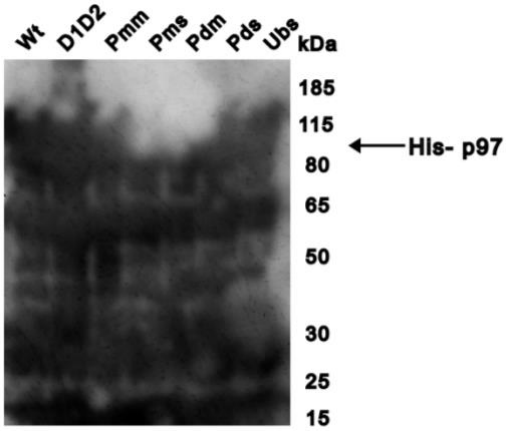

Figure 14: The His-tag western blot for all purified p97 mutant proteins in either elution buffer 250 mM (Wt and D1D2) or LFB1/50 (all other proteins). All were diluted to 3 mg/ml (same as Wt from new estimates, **Table 8**)

During the purification, the dialysis step proved to be sometimes fatal for the protein concentration. It was then considered if dialysis was necessary and if the proteins could stay in the elution buffer or if they needed to be dialysed into the extract friendly buffer LFB1/50. This was done by testing the elution buffers (250 mM and 500 mM imidazole) in egg extract in 1/8 and 1/10 extract volumes to see if replication capability of the extract was affected.

4.3. Testing *X.leavies* egg extract

The egg extracts were made and tested via the incorporation of radioactive dATP into newly synthesised DNA to see if and how well they had replicated. This was initially done with three time points with and without Ca^{2+} addition to release the extract into interphase. The extracts that showed good replication potential were then tested again with six time points to obtain a greater understanding on how they were replicating (**Figure 15 a**). Once the replicating extracts had been established, the buffers previously mentioned could be tested. **Figures 15 b and c**

shows the TCA replication assay results for these buffers. The first test was with LFB1/50 and 250 mM imidazole. **Figure 15 b** shows that although some samples were lost, the elution buffer with 250 mM imidazole was not affecting the ability of egg extract to synthesise nascent DNA. The next step was to see if the 500 mM imidazole affected DNA synthesis, and from **Figure 15 c**, the addition of 500 mM of imidazole was affecting replication. The conclusion was that the proteins can be kept in the 250 mM imidazole buffer but not in the 500 mM imidazole. So proteins eluted in 500 mM of imidazole would need to be dialysed. The proteins that required dialysis were Pmm, Pms, Pdm, Pds and Ubs. All the elution fractions were taken from the results in **Figure 12**. For all the dialysis conducted, F1 was used to be dialysed into LFB1/50, which is a buffer that has been shown not to affect DNA replication (Tarcan et al., 2022). Some of the protein samples were lost during dialysis, therefore, different size dialysis columns were tried (ranging from 2.5 kDa and 35 kDa). All proteins were then frozen in -80°C until needed.

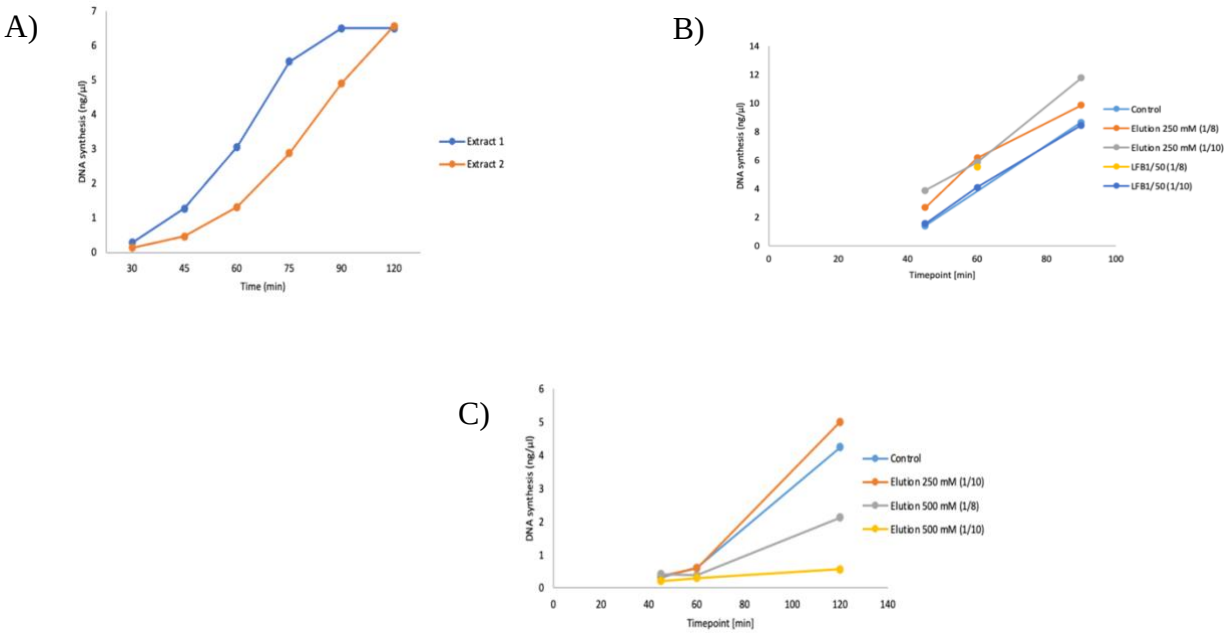

Figure 15: The DNA replication assay results for the egg extracts with no buffers added (**C**). **B** with LFB1/50, and elution buffer 250 mM imidazole. **C** with the addition of the elution buffer with 250 mM imidazole and 500 mM added to *X. leavies* egg extract at 1/10 and 1/8 dilutions. All experiments are n=1. The egg extract supplemented with de-membraned sperm nuclei, α-[P32]dATP and the DNA synthesis was measured at three preselected time points by measuring the amount of radiolabelled nucleotides that had been incorporated into the DNA

4.4. p97 interactions with chromatin associated proteins

Having purified all the proteins, the next step was to test to see if the mutations affect the ability of p97 to extract post-termination replisomes from chromatin. It has been shown previously that the addition of a high concentration of Wt p97 (or just LFB1/50 buffer alone) should not block replication and not impact replisome proteins such as Mcm7, Cdc45, PCNA or Psf2 unloading from the chromatin after replication has ended. However, the D1D2 mutant in the same buffer stops the unloading of other replisome proteins (Priego Moreno et al., 2014, Tarcan

et al., 2022). **Figure 16** shows the result from the experiment in which the D1D2 mutant behaves as expected (blocking unloading), however, the Wt and control (elution 250 mM) was also showing retention of replisomes on chromatin (**Figure 16 a**). These samples were then blotted for the His tag (**Figure 16 b**), which shows that there was a similar amount of protein added to the extract and there is a similar amount in the samples, although the D1D2 was a bit dirtier. This was repeated numerous times with different conditions tested such as changing the extract and the amount of protein added (ranging from 0.5 mg/ml to 0.3 mg/ml). However, all the tests showed the same result despite the differing factors.

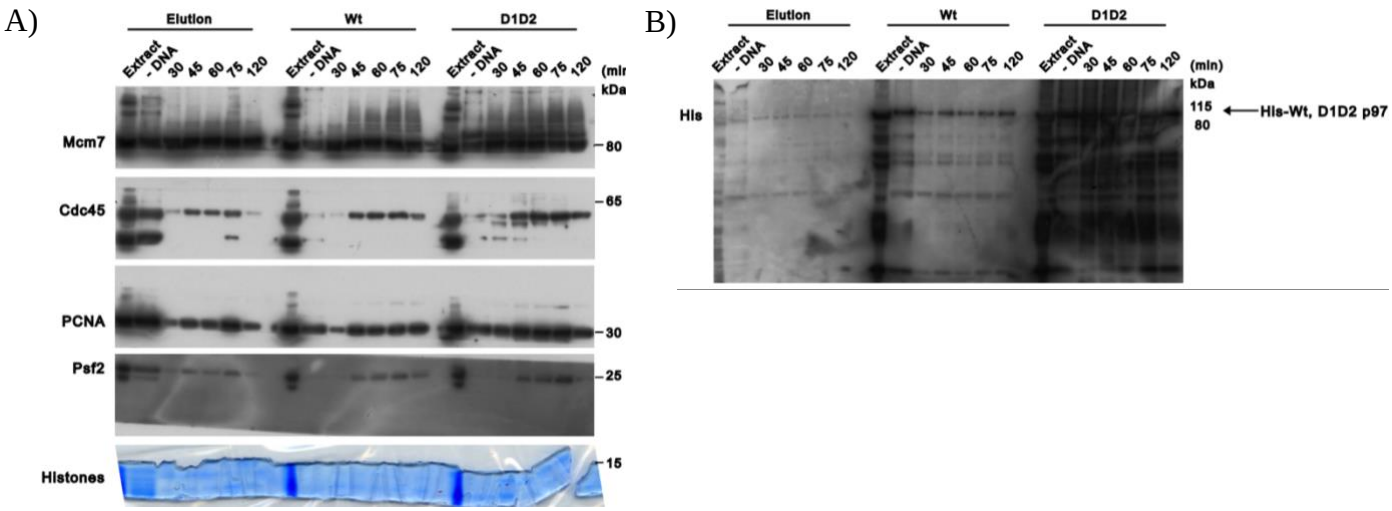

Figure 16: Chromatin isolation with elution buffer, Wt and D1D2 p97. Egg extract was supplemented with elution buffer with 250 mM imidazole (1/10 dilution), 0.5 mg/ml Wt in elution buffer and 0.4 mg/ml D1D2. At the time points identified, the chromatin was isolated. **A**, a western blot was carried out and was blotted for Mcm7, Cdc45, PCNA and Psf2. The histones were cut and developed with Coomassie. **B** was blotted with His-tag. This is a representative of 5 experiments performed. The histones are the loading control and if more than two bands are visible this indicates some contamination. A sample without DNA addition (-DNA) has been processed alongside other chromatin isolation and can be compared to the other samples as an chromatin specificity control). These is some contamination present in some of the -DNA samples (apparent from the bands in the chromatin) but not in the +DNA samples

As the replication proteins were retained on chromatin throughout the replication reaction, there was a possibility that the addition of the buffer/proteins was impacting the replication fork stability and unloading. **Figure 17** shows that the elution buffer and both Wt and D1D2 proteins did not affect DNA synthesis too much.

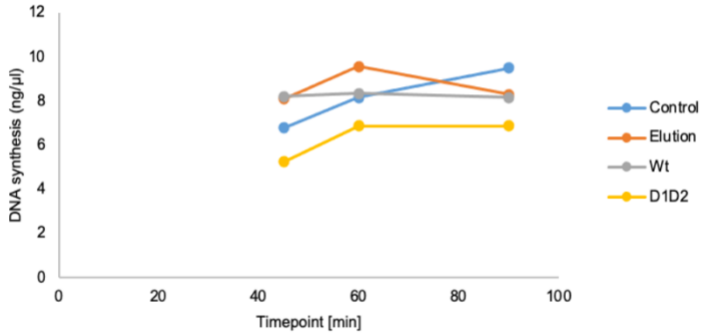

Figure 17: DNA synthesis assay with elution buffer (250 mM imidazole), Wt and D1D2 p97. The egg extract supplemented with de-membraned sperm nuclei, α-[P32]dATP and the DNA synthesis was measured at three preselected time points by measuring the amount of radiolabelled nucleotides that had been incorporated into the DNA. A 1/10 dilution (0.5 mg/ml) of elution buffer Wt and D1D2 p97 was added and replication was stopped at 45, 60 and 90 minutes. Representative of one experiment

DNA replication was not affected by the elution buffer with 250 mM imidazole and the proteins in this buffer (Wt and D1D2) as shown in **Figures 15 b, c** and **17**. However, this buffer and the proteins in this buffer were affecting the replisome protein dynamics (**Figure 16 a**). It was concluded that although dilution of the egg extract with this buffer does not affect the ability of the extract to synthesise DNA, it did affect unloading of proteins from chromatin after replication was completed, and therefore the elution buffer with 250 mM imidazole cannot be used in the experiments using egg extract to look at unloading of replisomes.

All of the other mutants were dialysed into LFB1/50, which is known to not affect the protein dynamics on chromatin. These mutants could then be tested to see if they impact DNA synthesis or block unloading of the replisomes as they are in a different buffer (Tarcan et al., 2022). However, as the Wt and D1D2 mutants are in a different buffer, these cannot be used in

the chromatin isolation as the positive and negative control, so just the LFB1/50 was used as the control. Unfortunately, **Figures 18 a** and **b** shows that although the LFB1/50 buffer did not affect the DNA synthesis ability of the extract, all of the mutant proteins were blocking or slowing down replication. This is also seen in **Figure 18 c** in the buffer, Cdc45 was present at replication forks at 60 mins but unloaded at 120 mins. However in all of the mutant protein samples, all chromatin associated proteins were all still on the chromatin at the late time point. Moreover, Mcm7 is accumulated in a polyubiquitylated form on chromatin in all mutant protein samples but not with the addition of a buffer at 60 mins. There is a possibility therefore that some of these mutants affect the ability of p97 to unload post-termination replisomes (ubiquitylated Mcm7). However, as the extract is not completing the DNA synthesis, it is also possible that the replication forks become stalled and were not completing replication termination. More research is needed to distinguish between these possibilities.

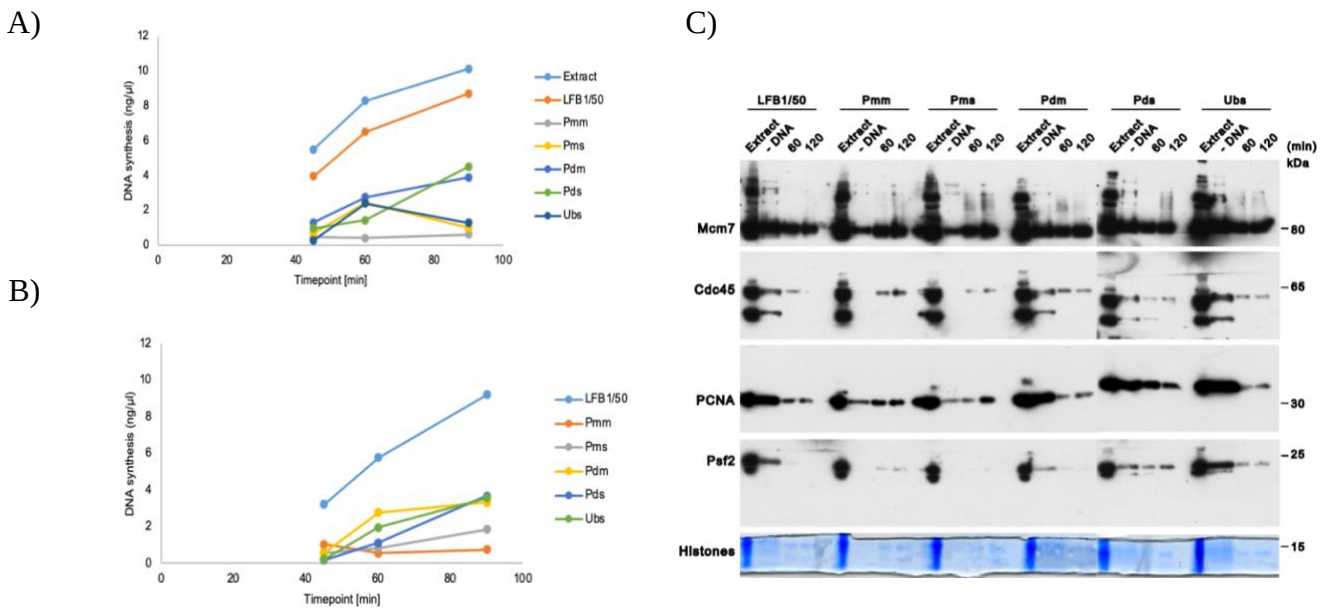

Figure 18: DNA synthesis assay and chromatin isolation with p97 mutant proteins. Egg extract added with the mutant proteins (in LFB1/50) or LFB1/50 in a TCA replication assay (**A** and **B**) and a chromatin isolation (**C**). All proteins were diluted to about 3 mg/ml (same concentration as new Wt estimate) and in **a** added at a 1/8 dilution (0.4 mg/ml final concentration) and for **b** and **c**, 1/10 dilution (0.3 mg/ml final). All n=1. In **c**, Several of the -DNA controls are contaminated as visible bands were present in histones loading control and also in the western blotting, however most of the +DNA samples are not contaminated from the two distinct histones bands in the loading controls. The chromatin was isolated at 60 and 120 minutes and blotted with the antibodies identified (**c**)

4.5. Cofactor interactions

It is known that p97 interacts with cofactors so it would be interesting to know if the mutations made changes the ability of the p97 protein to interact with its cofactors. To see if this was the case, his-pulldowns were carried out in five repeats, which were then incubated in the antibody for the cofactor to see the interactions. The cofactor proteins that were blotted for were Npl4, p47, Ubxn7 and Ufd1.

The first step was to get the optimal conditions using the Wt and D1D2 mutants. This was tested with different amounts of beads ranging from 30 µl per to 40 µl per sample (optimum 30 µl). Also testing to find out the minimum amount of beads that could be added to extract all of the his-tagged protein. Testing LFB1/50 with and without DTT and ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) was also carried out (no DTT and EGTA was optimum). This was done as the protein was still present in the depleted sample, so to see if removing the chelating compounds (DTT and EGTA) made any difference (which removing them stopped the protein being present in the depleted sample). Finally, the second wash carried out with LFB1/50 or with LFB1/200 and imidazole, to see if the addition of the extra imidazole and salt helps to clean the pulldown samples. It was found that LFB1/200 with 20 mM imidazole was optimal.

Four samples were taken at different stages of the experiment. An extract and input samples were taken to check that the recombinant protein was present in the samples and absent in the control (to detect any contamination). Depleted samples were taken to check that the protein had bound to the beads (no band should be present) and the pulldown sample to check that a similar amount of protein had been added, to check for unspecific binding.

Once the optimal conditions had been identified, all the mutant proteins were tested. **Figure 19** shows three repeats for all the mutants. The pulldown elution buffer samples that have bands present suggest that non-specific binding has taken place thus the results of these cannot be used for further analysis. For the mutants D1D2 and Pmm, the binding of the cofactors p47 and Ubxn7 is an example of this (**Figures 19 a**, **b** and **c**).

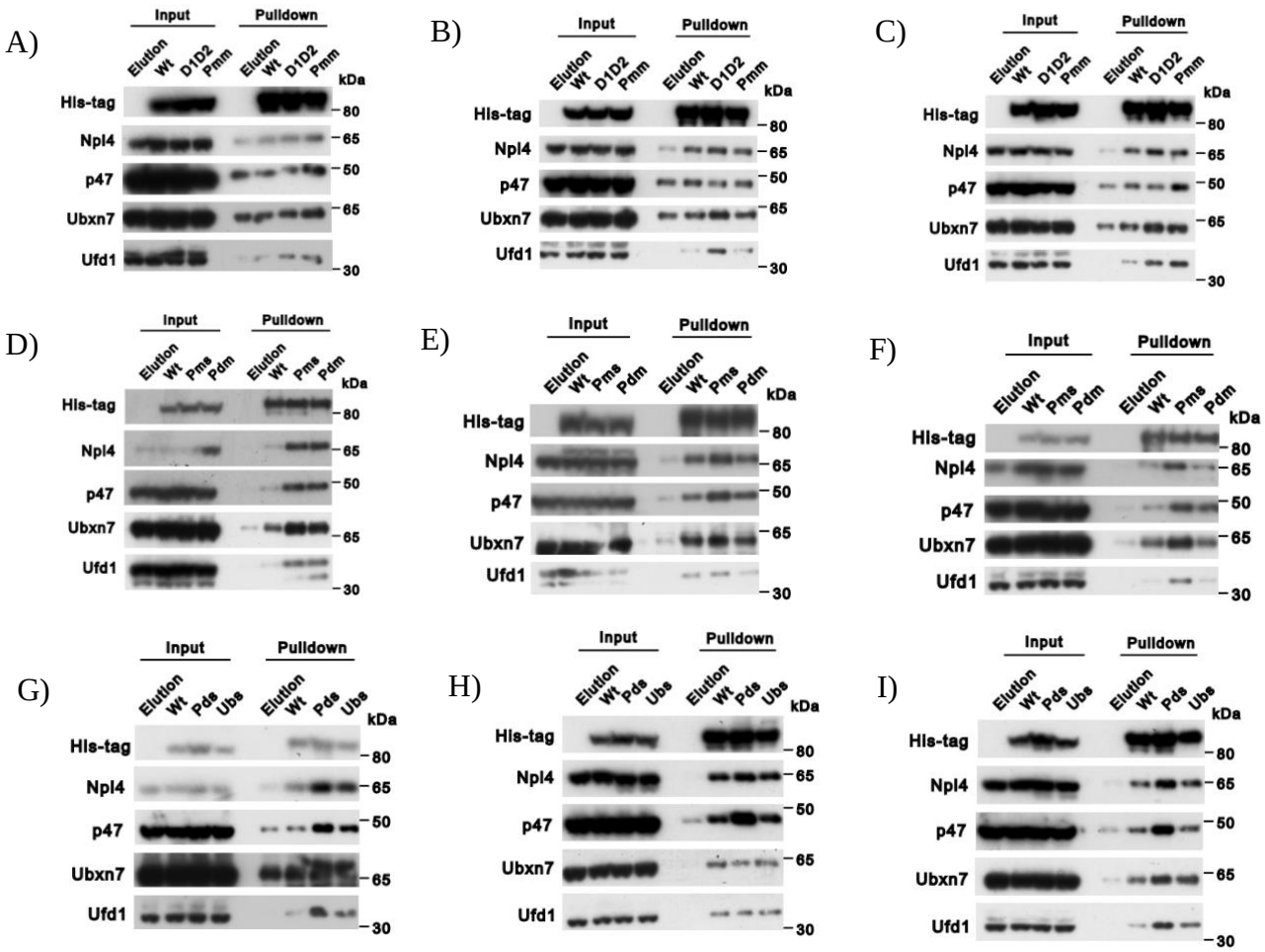

Figure 19: The western blot results for the his-pulldowns with mutant p97 proteins for three repeats, **A-C** with D1D2 and Pmm, **D-F** with Pms and Pdm and **G-I** with Pds and Ubs. All proteins were added at a 0.27 µg/µl final concentration. All contain Elution buffer with 250 mM imidazole and Wt as the controls. The first 4 lanes are the input and the second is the pulldown samples. The samples were blotted with the antibodies identified. This is the representative for 3 experiments for each mutant out of 5 which were performed.

Once all of the his-pulldown experiments had been completed, a statistical analysis could then be carried out, to see if there was any significant difference between the binding of the cofactors to p97 Wt and mutants. This was done by measuring the intensity of the bands by the number of pixels present in the bands. The mutant proteins were then normalised to the

His-tag blot (the level of immunoprecipitated p97 wt/mutant) and the fold change calculated in relation to the Wt (described in 3.5.).

As there was a lot of unspecific binding in the D1D2 and Pmm experiments for p47 and Ubxn7 (**Figures 19 a, b** and **c**) these have been omitted from statistical analysis. **Figure 20** shows that at a 95% significance level, the catalytically dead mutant may interact more with Ufd1, the phosphorylation mutations at nucleotides 7 and 9 (in S-phase) may interact more with Npl4 and p47, while the phosphorylation mutations at nucleotides 715 and 718 (M-phase) may increase interaction with p47 and Ufd1.

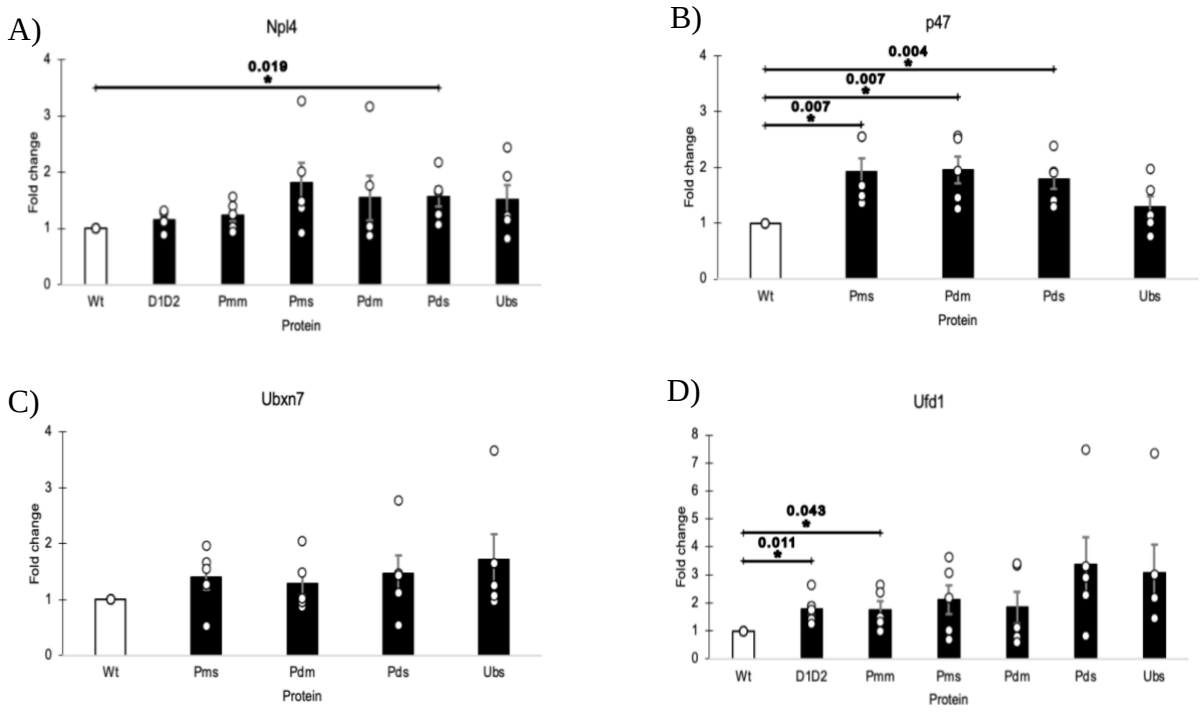

Figure 20: Bar charts with p97's cofactors interactions as fold change with Wt as 1 for five repeats. For **b** and **c** D1D2 and Pmm have been removed. Error bars with standard mean error of mean (SEM) shown and statistically significant results identified (95% confidence level) with a star and the p-value stated

4.6. Potential p97 substrates

The final part of this project was to identify new potential p97 substrates (proteins that are associated with chromatin and are polyubiquitinated) during DNA replication and how these behave when p97 is blocked. These experiments are a good source of identifying potential p97 substrates as they identify all of the proteins that are present in the chromatin sample with or without p97 inhibition. A IP, his-pulldown or a p97i chromass was performed by a previous member of the lab and the samples were sent off for mass spectrometry. This identified all proteins present in the samples. It could also determine if there had any potential ubiquitination sites present on the protein (G-G remnant after Trypsin digestion) which is useful to note as p97 binds to ubiquitinated proteins (Bodnar and Rapoport, 2017).

The first step was to analyse mass spectrometry data from IP, His-pulldown and p97i chromass in S and M phases of the cell cycle in normal conditions and when p97 was inhibited, to identify potential proteins that could interact with p97. An IP is when a specific antibody is added to bind to a specific protein (in this case p97), and all of the other proteins that it is attached to (e.g. cofactors) are also extracted. A His-pulldown uses a similar principle but uses beads that attach to a his-tagged protein (His-Ubiquitin in this case to see which proteins have ubiquitin attached, thus could potentially interact with p97). A p97i chromass is similar to a His-pulldown but is not enriched for ubiquitinated proteins.

The IP experiment in S- phase was performed during replication termination, the replicating egg extract had NMS-873 added to obtain a large amount of replisomes on the chromatin. At 60 mins, most of the replication had occurred so replication was stopped. Benzonase was added to digest the DNA, releasing the proteins. IgG beads were also added which were p97 specific and non- specific. A western blot was carried out and showed that p97 and Npl4

could be drawn from the chromatin. An SDS-PAGE was carried out and the gel was sliced and analysed via mass spectrometry to get more detail.

In the S-phase His-pulldown, the chromatin was allowed to replicate with the addition of NMS-873 (to allow the build-up of proteins on the chromatin). His6-Ubi was added so any proteins that are usually ubiquitinated, will have the modified ubiquitin which can be detected later. Replication was stopped in late S-phase with ANIB/100 and iodoacetamide (GE Healthcare). The chromatin was pelleted and the buffer was removed. It was then resuspended in ANIB/100, iodoacetamide and Benzonase (Sigma) to digest the DNA. It was then denatured with a Urea buffer and mixed with pre-washed His-Tag isolation and pulldown Dynabeads (Life Technologies). The beads were washed and boiled, then a gel was ran and sent off for mass spectrometry.

The proteins had to have at least two times total enrichment to be considered in both the IP and pulldown experiments. Enrichment was worked out as the identified protein in the test (with p97i added) having two times more peptides identified than the control (no p97i added). **Table 9** has the proteins identified in both the IP and His-pulldown for S-phase. Proteins that were identified to be contaminators such as keratin were not included, outlined in 3.6.1..

Table 9: The proteins identified to be present in both IP and His-pulldown experiments in S-phase of the cell cycle and to have at least two times enrichment. Their name, abbreviation, size (kDa), number of peptides identified and the total enrichment calculated stated. For enrichment calculation, when 0 peptides were identified in control, 1 was used instead to be able to calculate the enrichment.

		Immunoprecipitation of p97			His-pulldown of His-Ubi		
Protein	Size (kDa)	Number of peptides		Total enrichment	Number of peptides		Total enrichment
		Control	p97i		Control	p97i	
Replication protein A 70 kDa DNA-binding subunit (RPA1)	67	29	149	5.1	73	536	7.3
Proliferating cell nuclear antigen (PCNA)	29	19	54	2.8	6	48	8
DNA replication licensing factor mcm7-B	82	0	42	42	54	360	6.7
FFA-1	161	0	31	31	309	639	2
Zona pellucida C glycoprotein (xlZPC)	50	2	28	14	0	20	20
MGC79017 (RPA2)	29	8	27	3.3	0	43	43
ATP synthase subunit alpha (ATPa1)	60	0	25	25	2	93	46.5
Ubiquitin carboxyl-terminal hydrolase (Usp5)	93	0	18	18	0	12	12
Lzpb-a protein (Zp4)	60	0	16	16	9	57	6.3

Gp37	59	0	15	15	9	64	7.1
MGC80116 (Trafd1)	68	0	11	11	0	33	33
LOC414552 (Tax1bp1)	89	0	11	11	0	12	12
DNA topoisomerase 2-binding protein 1-A (Topbp1)	169	0	11	11	6	160	26.7

Having identified the proteins enriched in both experiments. The next stage was to investigate if any of them, or any other proteins had any mapped ubiquitylation sites. **Table 10** has the proteins with ubiquitin sites from S-phase in the same experiment from **Table 9**. Only the abbreviations were used. To identify a ubiquitin site, the whole protein sequence was looked at and if a K was identified, it had to have a 95% confidence that this was the right amino acid. The IP did not identify any proteins while the his-pulldown identified five proteins. One protein that was identified twice was RPA.

Table 10: The proteins identified in the IP and His-pulldown in S-phase with the ubiquitin sites. The number of peptides in the control and ubiquitin sites identified, total enrichment of this and the amino acid number that ubiquitin could bind at a 95% confidence level were all noted. The abbreviation of each protein was used

	Immunoprecipitation of p97				His-pulldown of p97			
Protein	Number of peptides		Total enrichment	Ubiquitylated lysines (95%+)	Number of peptides		Total enrichment	Ubiquitylated lysines (95%+)
	Control	Ubiquitylated lysine's			Control	Ubiquitylated lysine's		
RPA1					73	536	7.3	88, 157, 174, 235, 258, 358, 401, 544
PCNA					0	1	1	164
RPA2					0	3	3	88, 237
ATPa1					0	3	3	167
Topbp1					0	3	3	675, 1415

Next, the experiments that were carried out in M-phase were analysed. In M-phase the IP involved de-membraned sperm being added to egg extract in interphase, with the addition of MLN4924 (a Cul2 inhibitor) to block replisome disassembly. Caffeine was added to gain a high amount of chromatin bound proteins and NMS-873 to keep p97 on the chromatin. After 90 mins of incubation at 23°C, DNA replication had ended and cyclin A1Δ was added to start mitosis. The chromatin was isolated after 30 mins when most of the p97 had accumulated and the proteins on chromatin were released. The protein complexes were agitated by placing the samples in a diagenode cold water sonicator for 5 mins with the following settings: medium

setting with 15 s on and 15 s off. Potassium acetate was added to a 300 mM concentration to increase the solubility. The samples were then mixed at 4°C for 2 h with Epoxy beads (Dynabeads M-270 epoxy bound to antibodies). At 4°C and with rotation, the beads were then washed 5 times: 2 times with ANIB100, then with LFB1/50 with 0.1 % Triton X-100, and then twice with ANIB100. The material that has been immunoprecipitated was boiled in NuPAGE LDS loading buffer for 5 mins, then vortexed and boiled again for 5 mins to elute the material. The sample was analysed by mass spectrometry as explained in (Sonneville et al., 2017) with collaboration of Dr Richard Jones from MS Bioworks LLC.

In M-phase the other experiment that was used for the data analysis was a p97i chromatin mass spectrometry (chromass). DNA replication was allowed to occur in egg extract and NMS-873 was added to block replisome disassembly. A high concentration of Cyclin was added to make the cell enter M-phase. The chromatin was then isolated and the whole sample was sent off for a mass spec analysis.

The data for the M-phase experiments was analysed, collected and displayed in **Table 11**. This looked at protein interactions and proteins that are associated with chromatin. Eight proteins were identified to be enriched in both experiments.

Table 11: The proteins identified in M-phase of the cell cycle when testing for protein interactions (IP) and proteins on chromatin (chromass). The proteins name (with abbreviation) shown with its size (kDa), number of peptides in the control and p97 and the total enrichment in both conditions

		IP			p97i chromass		
Protein	Size (kDa)	Number of peptides		Total enrichment	Number of peptides		Total enrichment
		Control	IP		Control	Pulldown	
MGC154888 (Zp4)	59	0	129	129	0	12	12
Gp37	59	27	123	4.6	0	23	23
Lzpb-a (Zp4)	60	24	113	4.7	0	30	30
XlZPA (Zona pellucida sperm-binding protein) (Zp2)	78	3	59	19.7	0	10	10
Elongation factor 1-alpha (Eef1ao)	50	0	42	42	11	28	2.5
Elongation factor 1-gamma-B (Eef1g-b)	50	0	38	38	0	14	14
LOC494816 (Npl4)	69	5	21	4.2	0	27	27
Npl4	69	3	16	5.3	0	22	22

The next step was to look at the proteins with K residues like in S-phase, however, none of the proteins identified in **Table 11** had any K residues with at 95% confidence level or above so no table was made.

When the proteins in **Tables 9, 10** and **11** were analysed, some of the proteins were not involved in DNA replication such as Eef1g-b which is involved in translation (Sammaibashi et al., 2018). Others are cofactors of p97, such as Npl4 (Tarcan et al., 2022). Others such as Gp37 protein is not associated with *Xenopus*, but is part of a bacteriophage (Bartual et al., 2010). It would be expected that p97 substrates that are important during the cell cycle and would have DNA related roles. This leaves, topbp1, PCNA, Mcm7, RPA and FFA-1 (referred to as WRN). Due to time constraints, only some could be looked into thus the most interesting ones were picked. RPA and WRN were chosen as there is less research between the links of p97 and these proteins.

Now that the proteins had been identified, they could then be tested to see if they have any interactions with p97. This would be done by performing a chromatin isolation with normal DNA replication and with a p97i added. This could then be blotted with an antibody for the potential protein candidate (RPA or WRN) to see if there is any more or less polyubiquitination of these proteins when p97i was added.

4.6.1. RPA ubiquitination

As serum with antibodies against RPA was available in the lab, it was used to blot with after chromatin isolation experiments had been carried out in normal conditions (with DMSO added) and when a p97i was added. **Figure 21** shows three repeats of this experiment. It seems like the polyubiquitination (smaller bands between the subunit bands) in both conditions was fairly similar across all repeats. Upon closer inspection, there could be slightly more in the p97i repeat 2 (**Figure 21 b**), but it is not obvious.

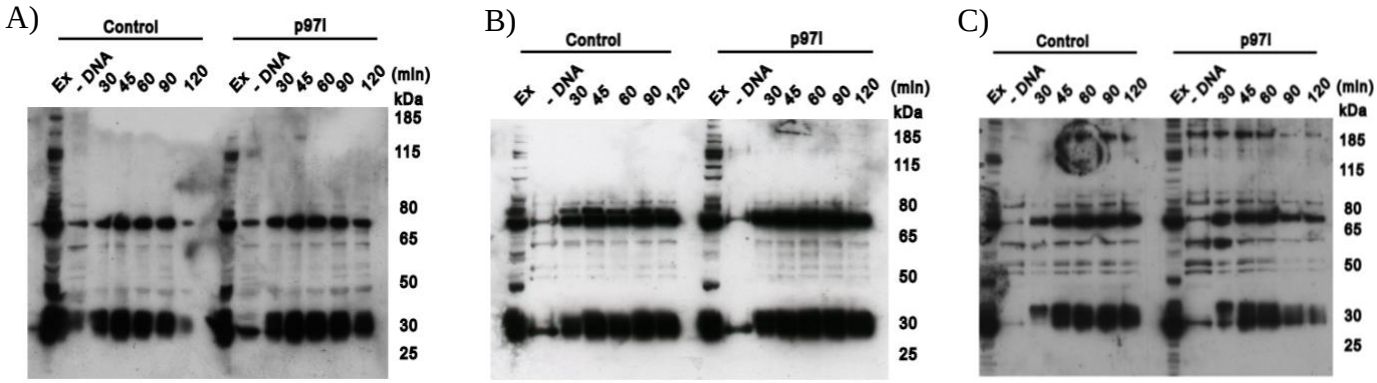

Figure 21: RPA and p97i chromatin isolations when the chromatin was isolated at the time points identified. Three repeats from chromatin isolations with DMSO (control) and NMS873 (p97i) added at a final concentration of 50 μm and blotted for RPA 70 and 32 kDa subunits

The other protein that was identified and chosen to be a potential candidate for interaction with p97 during DNA replication was WRN, however there was no purified antibody or sera that has been tested, thus the first step was to test the sera to see if the antibodies in the sera could recognise WRN. If it could, then the sera could be used for blotting or the antibody may had needed to be purified.

4.7. WRN IP

An IP using two rabbit sera was performed to see if the antibody can recognise a band of the size of WRN (180 kDa), if it can immunoprecipitate a band of 180 kDa, and if the antibodies needed purifying or if the sera can just be used for western blotting. In both rabbit sera's, there is a strong band at around 50 kDa in the IP samples of the egg extract when pre-sera and sera was added (**Figure 22**). However, the WRN protein is 180 kDa (Muftuoglu et al., 2008), so this could be the antibodies heavy chain. There is no obvious band in the IP for both rabbits' sera's, however, there may be a slight band in the input, so it may identify WRN. However, as

the band was not obvious, the antibodies could be purified and see if they could pick up the WRN protein once they had been purified.

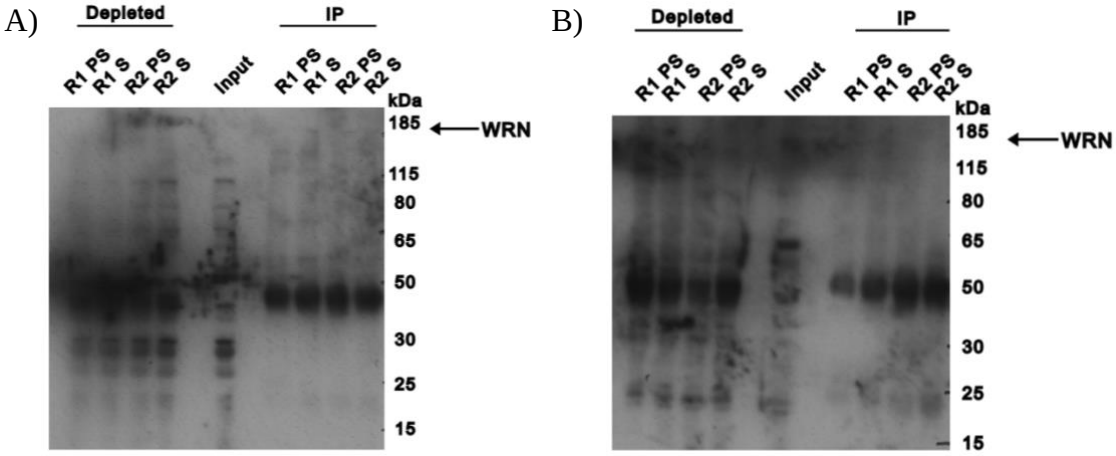

Figure 22: The IP of egg extract of WRN. It was blotted with sera from rabbit 1 sera (**A**) and **B** with rabbit 2 sera. R1 is rabbit 1, R2: rabbit 2, PS: pre sera and S: sera. Both representative of one experiment

4.7.1. WRN purification

To purify the antibody, an affinity column needed to be made containing the purified antigen. The WRN protein needed to be expressed, purified and dialysed into a coupling buffer with a concentration of about 1 mg/ml. The purification of WRN was quite dirty as there are many bands present as well as the one at 65 kDa (**Figure 23 a**). The dialysis did clean it up slightly (**Figure 23 b**), however, it is not at the required 1 mg/ml, so different conditions will need to be tested to get the optimum conditions for purification.

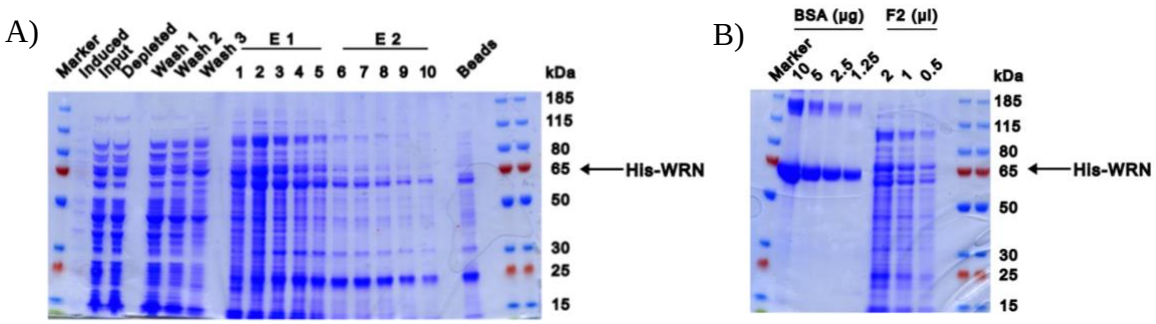

Figure 23: SDS page gel of WRN purification and BSA gel. **A**, with the WRN purification showing the induced, input, depleted, 3 washes, 10 elution's and beads. **B** showing the BSA of fraction 2 of a after dialysis in the coupling buffer

More purifications would have been carried out to find out the optimum conditions, however, due to time constraints, this was not able to be completed.

5. Discussion

5.1. Protein purifications

For p97, all but one of the plasmids for the mutants (except Ubm) were produced via SDM. These were then used to express and purify the mutant proteins, (although it did take a while to get the required concentration of 4 – 5 mg/ml). **Figures 12 a- g** shows these purifications and **Figures 13 a- e** and **Table 8** show the concentrations of the proteins. The buffer that the proteins were dissolved in contained imidazole. After testing, it was found that although at a 250 mM of imidazole, it did not affect DNA synthesis, but it did affect the protein dynamics in terms of interaction with chromatin, while 500 mM affected replication (**Figures 15 b, c** and **16 a**). This meant that the proteins needed to be dialysed into LFB1/50 which is a buffer that doesn't affect replication or protein dynamics shown in **Figures 15 b**, **18 c** and the paper by (Tarcan et al., 2022).

5.2. Unloading of chromatin associated proteins

It was shown previously that adding Wt p97 to egg extract does not affect DNA replication and the dynamics of replication proteins association with chromatin. While D1D2 p97 mutant was shown to block proteins unloading but not DNA replication (Priego Moreno et al., 2014). The other mutants' behaviours were unknown. However, the proteins made for these experiments, the Wt and D1D2 did not affect DNA synthesis (**Figure 17),** but they both affected the protein dynamics, especially Mcm7 unloading (**Figure 16 a**). This could be explained by the fact that both proteins were in the elution buffer that affected protein dynamics. These observed effects are therefore most likely not due to protein added but the components of the elution buffer.

All of the other mutant p97 proteins were in LFB1/50 which doesn't affect DNA synthesis or protein dynamics. However, when added to egg extract they all affected DNA synthesis and protein dynamics as shown in **Figures 18 a – c**. It is possible that this is a true reflection of their function, but, there was a lot of truncated, inactive protein in the solution (**Figures 12, 13** and **14**). This accounted for about 50 to 75% of the total protein, which was also being added to the egg extract. This could have potentially been impacting replication and protein dynamics, so if the purifications were carried out again, a different technique could be used such as a different type of chromatography e.g. the AKTA system to see if a cleaner sample can be acquired. The mutants were not affecting replication in exactly the same way as in **Figures 18 a** and **b**, Pmm showed no replication, while Pds showed some slow replication. So other explanations could be that the proteins were frozen for too long, they had precipitated or the pH of the buffer could have been slightly wrong. Taking everything into consideration, it seems most likely there was an issue with the mutant proteins (Pmm, Pms, Pdm, Pds and Ubs) that were made and there was an issue with the buffer that the Wt and D1D2 proteins were in. As a result, further testing is required to understand the protein dynamics for the p97 mutants.

5.3. p97 Cofactor interactions

The interactions of the p97 mutants and its cofactors present in the egg extract were also analysed. The catalytically dead mutant may cause p97 to interact more with Ufd1. The phosphorylation mutations at nucleotides 7 and 9 (S-phase) may cause p97 to interact more with Npl4 and p47. The phosphorylation mutations at nucleotides 715 and 718 (M-phase) may increase p97's interaction with p47 and Ufd1 (**Figure 20**). Currently there have been no known studies looking at the specific mutations that were made at nucleotides 7, 9, 305, 578, 715 or 718. However, a study suggests that mutations in the N- terminus region (between nucleotides

1 and 187) may impact p97 binding to its cofactors and its substrates (Mori-Konya et al., 2009, Wang et al., 2004).

p47, Npl4 and Ufd1 bind to the N-terminus of p97 as shown in **Figure 24** and there does seem to be changes in interactions between the binding of Npl4 and p47 when mutations in this area are present. This could be due to the mutations causing a slight conformational change in this area, and possibly making it easier for these cofactors to bind. This pattern is also shown in a study that mutated amino acid number 155 and the two cofactors Ufd1 and Npl4 bound tighter to p97 (Beskow et al., 2009, Blythe et al., 2019, Conicella et al., 2020)

The M-phase phosphorylation mutations in the D2 domain (715 and 718) also change the interactions between p97 and its cofactors p47 and Ufd1, but they do not bind in these areas. The mutations are quite close to the N-terminus region so could potentially affect the structure of this region. The mutations could change the folding of the α-helix and could move it closer to the N-terminus, however, no studies have looked into if mutations in the D2 region impact the binding of cofactors to p97 (Beskow et al., 2009, Blythe et al., 2019, Conicella et al., 2020).

The catalytically dead D1D2 mutant also affected the binding of Ufd1 to p97. Ufd1 also does not bind to this region. However, as the mutations are located in the conserved region, this could cause a conformational change that could affect the whole structure of p97, thus affecting its ability to bind to cofactors (Beskow et al., 2009, Conicella et al., 2020, Le et al., 2016). It could also be due to the fact that p97 uses ATP as its energy to function (which causes changes in its structure), and as the mutation causes it not to be able to convert ATP into ADP, the structure does not change, and some cofactors bind to p97 better in different states (ATP or ADP present). As a result, this could cause differences in cofactors binding. It has been found

that when ADP is bound, p32 and p47 binds less to p97 than when ATP is bound, however, in **Figures 19 a- c** and **20 b**, there is no change in p47 binding (Bulfer et al., 2016).

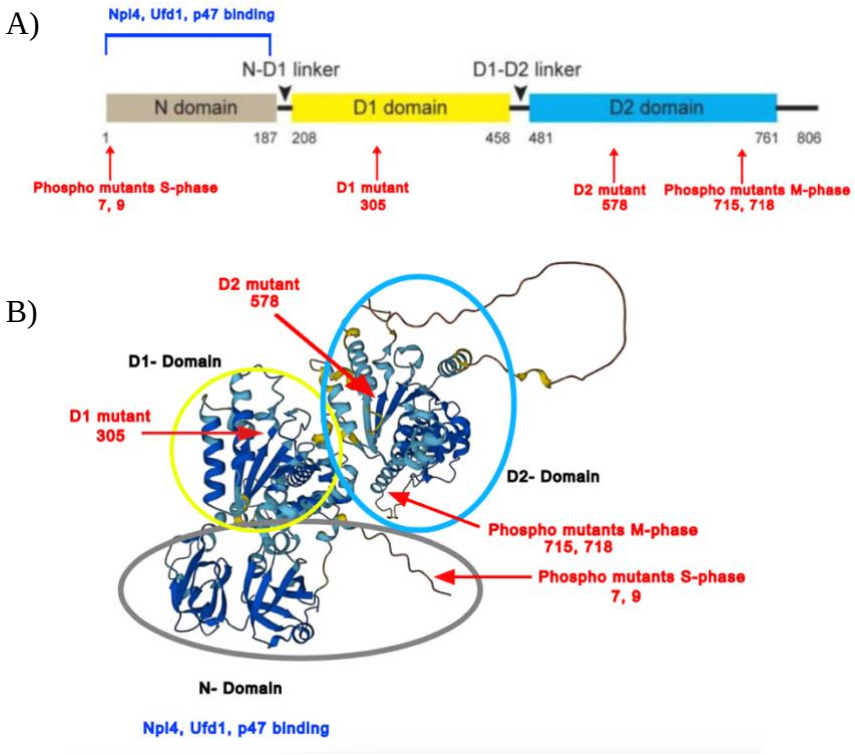

Figure 24: p97 structure, mutations and cofactor binding. A 1D diagram (**A**) and 3D diagram (**B**) of p97 stating where the mutations of mutants are located in red, the domains (grey- N-domain, yellow - D1 domain and light blue D2 domain) and where Npl4, Ufd1 and p47 binds (dark blue) (Conicella et al., 2020, Jumper et al., 2021, Le et al., 2016, Varadi et al., 2022, Xia et al., 2016)

It is also important to note that Ufd1 and Npl4 are involved in removal of proteins from chromatin, while p47 is not associated with this process but with vesicle fusion to membranes (Fujisawa et al., 2022, Kondo et al., 1997). As p47 was identified in these experiments, more

tests could be carried out to investigate this further to see how else p97 and p47 interact during DNA replication.

From **Figure 20**, it is also worth noting that the mutations only seem to affect the binding of p97's major cofactors that were tested (Ufd1, Npl4 and p47) and not its minor cofactors (Ubxn7). As the major cofactors are involved in which function p97 needs to carry out, for example chromatin related function or ERAD functions, perhaps the post translation modifications also play a role in which major cofactor needs to bind at a particular time in order to carry out a certian function. However, more experiments will need to be carried out to see if this is the case (Park et al., 2017, Tarcan et al., 2022, Yuan et al., 2001).

It is also important to look at the differences in p97's function and its cofactors in S and M phases of the cell cycle as there are some slight differences in how it works. p97 recognises different ubiquitin K linked chains, in S-phase it recognises K48 linked chains, while in M-phase it recognises K6 and K63 linked chains, thus, p97 could be regulated differently (Tarcan et al., 2022). Firstly, there are some differences in PTMs in S and M phases shown in **Figure 11** with different amino acids being phosphorylated, ubiquitinated and acetylated. As previously mentioned, the phosphorylation and ubiquitin mutants' functions could not be determined thus it is unclear if these PTM's help regulate the differences between the two phases. However, it can be seen that the phosphorylation may impact the binding of cofactors. In S-phase, it may increase binding of Npl4 and p47, while in M-phase may increase interaction of p47 and Ufd1 (**Figure 20**).

5.4. Potential p97 substrates

A few different proteins were identified that may interact with p97 during DNA replication from **Tables 9-11**. One being Mcm7, which is known to interact with p97 and be polyubiquitinated (Fullbright et al., 2016). Another is RPA which is a single stranded binding (SSB) protein, where all of its functions come from its ability to bind ssDNA. Some of its functions include, aiding in loading the polymerases to the DNA, aiding in the DNA unwinding and DNA repair (Bell and Dutta, 2002, Iftode et al., 1999, Wold and Kelly, 1988). RPA can be ubiquitylated, with many different ubiquitin chain types, on its 32 subunit (RPA32) on residue 23 in S-phase and 29 in G/M-phase (Dubois et al., 2017). When it is polyubiquitinated during DNA damage, it can be removed from chromatin via p97 (Inano et al., 2017, Mirsanaye et al., 2021). As it has been identified in the IP, His-pulldown, p97i chromass and in numerous papers for its potential interaction with p97, RPA needed to be investigated further.

WRN was also identified and is a RecQ helicase protein that can unwind DNA. It has many different functions including involvement in DNA replication and DNA repair (Mukherjee et al., 2018). During DNA stress, WRN is phosphorylated (via the ATR-mediated response), which then causes it to be ubiquitinated via Phosphorylation-dependent ubiquitination. WRN can then be removed from chromatin via p97 and degraded via the 26S proteosome system (Partridge et al., 2003, Rodríguez Pérez et al., 2023, Su et al., 2016). Like RPA, the interactions between WRN and p97 need further investigation.

The analysis of a chromatin isolation when p97i was added showed that there was no obvious difference in the polyubiquitination of RPA compared to when no p97i was added in S-phase (**Figure 21**). However, RPA is heavily involved in DNA damage as it binds to the ssDNA and recruits other proteins to aid with the repair. When this occurs, the replisomes may still be

present on the chromatin in M-phase (although DNA replication has stopped) (Moreno and Gambus, 2020, Mukherjee et al., 2018). So further experiments could be carried out in M-phase to see if there are any differences.

There was no WRN antibody available in the lab so the sera were tested and the antibodies could tried to be purified (**Figure 22**). The WRN protein therefore needed to be purified at a 1 mg/ml concentration, however, in **Figure 23** it the concentration was not reached and due to time constraints this could not be taken any further.

The proteins that were identified to contain ubiquitin sites were RPA subunits 1 and 2, PCNA, ATPa1 and Topbp1 (**Table 10**). The ATPa1 is a protein that is found in the mitochondria (Wang and Gengenbach, 1990), so this is from contamination. From these results eight ubiquitinated lysine's were identified on RPA1, however, a study identified fourteen ubiquitinated lysine's and the only one that was the same, was K88. This study also identified two ubiquitinated lysine's on RPA2 (which was also identified in this analysis), however, the lysine's were different (36 and 37 in the paper and 88 and 232 in **Table 10**) (Elia et al., 2015). For the RPA1 subunit, 85% of the ubiquitin sites identified in the paper were also identified on a protein modification database for human cells (Inc, 2024). The study used Henrietta Lacks (HeLa) cells which are human cells, this could be why there are some differences in the ubiquitinated sites identified in the paper to the mass spec data that was analysed in this project. To make sure that the ubiquitin sites for RPA in *Xenopus* are the ones stated in **Table 10**, more repeats could be carried out.

PCNA was also identified to be ubiquitinated and K164 was identified to have a ubiquitin attached in **Table 10**. PCNA can be mono or polyubiquitinated (with K63 chains) at this site

via a E3 ligase when there is DNA stress. p97 has also been shown to bind to PCNA and aid with its degradation when a ubiquitin chain is attached. However, p97 may be inhibited by Chromosome 1 Open Reading Frame 124 (C1orf124) when PCNA is needed to stay on the chromatin for repairs (Davies et al., 2008, Ghosal et al., 2012, Leach and Michael, 2005) . As numerous studies suggest that PCNA is ubiquitinated on K164 and the mass spec analysis also says this, it is likely that this is the case.

Finally, **Table 10** indicated that Topbp1 was ubiquitinated and a study has also shown this but no studies have identified the exact number of sites or the position/s of this so it is possible that the positions identified could be correct, but further analysis will need to be conducted to ensure this is the case (Kim et al., 2021).

The use of p97 inhibitors have been used clinically such as 1-[4-(benzylamino)-5H,7H,8H-pyrano[4,3-d]pyrimidin-2-yl]-2-methyl-1H-indole-4-carboxamide (CB-5083), that was used in clinical trials for myeloid leukaemia (AML). CB-5083 impacts p97's D2 domain, however it does not affect all of its functions. It was later found that some of p97 cofactors interfere with the inhibitors function such as the presence of p47 resulted in a three-fold decrease in the inhibitors function when it was present. So p47 was also making the inhibitor less effective. It was also found then Npl4 and Ufd1 had little effect on the inhibitors function (Her et al., 2016, Kilgas and Ramadan, 2023, Tang et al., 2019). As p97 cofactors can affect inhibitors, it is vital that more research is carried out on p97s cofactors and its inhibitors to see if new drugs could be effective for certain conditions such as cancer.

6. Future work

As many of the experiments did not work or did not get completed such as the chromatin isolations with the mutant p97's and testing for WRN when p97i is added, these will need to be completed. Once all of these experiments have been carried out, links between the PTM's on p97's function and binding to cofactors can be drawn. More PTM mutants could be made such as the acetylation mutants and see if these affect p97's function and the cofactors ability to bind to p97 by carrying out chromatin isolations and His-pulldown experiments in the same way as outlined in the methods.

7. Conclusion

To conclude, phosphorylation of p97 in S-phase may increase interaction with Npl4 and p47, while phosphorylation in M-phase may increase interaction of p97 with p47 and Udf1. The catalytically dead mutant may also increase interaction with Ufd1. Majority of the p97 mutants that were made, blocked replication and stopped the unloading of chromatin associated proteins, thus they need to be made again and experiments repeated. RPA and WRN were identified as potential candidates for proteins that could be p97 substrates during DNA replication. During S-phase, there is no difference in the interactions between p97 and RPA, but further testing could be carried out in M-phase to see if there are any differences. Finally, the WRN antibodies need purifying from the sera and could be used in chromatin isolation experiments with normal conditions and when a p97i is added to see if there are any differences in p97 and WRN interactions.

8. List of abbreviations

A	Adenine
A	Alanine
AAA	ATPases Associated with diverse cellular Activities
AML	Myeloid leukaemia
AMP	Adenosine monophosphate
ANIB100	Acetate Nuclear Isolation Buffer
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATPa1	ATP synthase subunit alpha
ATR	Rad3-related
ATX3	Ataxin 3
bp	Base pairs
BSA	Bovine Serum Albumin
C	Cytosine
C1orf124	Chromosome 1 Open Reading Frame 124
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CB-5083	1-[4-(benzylamino)-5H,7H,8H-pyrano[4,3-d]pyrimidin-2-yl]-2-methyl-1H-indole-4-carboxamide
Cdc45	Cell division control protein 45
CDC48	Cell division control protein 48
Cdc6	Cell Division Cycle 6 protein
Cdt1	Chromatin licensing and DNA replication factor 1
Chromass	Chromatin mass spectrometry
CHX	Cycloheximide
CMG	Cdc45-Mcm-GINS
$CUL2^{LRR1}$	Cullin 2 leucine rich repeat protein 1
CPD	Cyclobutane pyrimidine dimer
Cys	Cysteine
D	Aspartic acid
D1D2	Catalytically dead mutant or ATPase mutant
DMSO	Dimethylsulfoxide
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleoside triphosphates
DSB	Double strand break
DTT	Dithiothreitol
DUBs	Deubiquitinates
E	Glutamic acid
E1	Elution buffer 1
E2	Elution buffer 2
EDTA	Ethylenediamine tetraacetic acid
Eef1ao	Elongation factor 1-alpha
Eef1g-b	Elongation factor 1-gamma-B
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ER	Energy Regenerator
ERAD	Endoplasmic reticulum associated degradation
F	Fraction

Faf1	Fas Associated Factor 1
Fen1	Flap endonuclease 1
G	Guanine
G	Glutamine
G1-phase	Growth 1 phase
Gly	Glycine
h	Hour
H_2O	Water
HeLa	Henrietta Lacks
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
Ile	Isoleucine
IP	Immunoprecipitation
IPTG	Isopropyl β- d-1-thiogalactopyranoside
IR	Ionising radiation
K_3PO_4	Potassium phosphate
KCl	Potassium chloride
KoAc	Potassium acetate
KOH	Potassium hydroxide
LB	Luria Bertani broth
Leu	leucine
Lys or K	Lysine
M-phase	Mitosis phase
Mcm2-7	Minichromosome maintenance complex 2-7
Mcm7	Minichromosomal maintenance complex component 7
MetII	Metaphase II
$MgCl_2$	Magnesium Chloride
MgOAc	Magnesium acetate
Min	Minute
MMR	Marc's modified ringer's buffer
MOPS	1x 3-(N-morpholino) propanesulfonic acid
Na_3PO_4	Sodium phosphate
$Na_4P_2O_7$	Sodium pyrophosphate
NaAzide	Sodium azide
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
Ni-NTA	Nickel-nitrilotriacetic acid
NMR	Nuclear Magnetic Resonance
Npl4	Nuclear Protein Localization 4
OD	Optical density
ORC	Origin Recognition Complex
p97i	p97 inhibitor
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
Pdm	Phosphorylation Dead M-phase
Pds	Phosphorylation Dead S-phase

Phe	Phenylalanine
Pmm	Phosphorylation Mimicking M-phase
Pms	Phosphorylation Mimicking S-phase
PMSF	Phenylmethylsulfonyl fluoride
Pol	Polymerases
Polδ	Polymerase delta
Polε	Polymerase epsilon
Pre-RC	Pre-replicative complex
PS	Pre sera
PTM	Post translational modifications
PVDF	Polyvinylidene difluoride
R	Arginine
R1	Rabbit 1
R2	Rabbit 2
RNA	Ribonucleic acid
RNase H1	Ribonuclease H1
RNF8	RING finger protein 8
RPA	Replication protein A
RPM	Revolutions per minute
RT	Room temperature
s	Seconds
S	Serine
S	Sera
S-phase	Synthesis phase
SDM	Site direct mutagenesis
SEM	Standard mean error of mean
Ser	Serine
SOC	Super optimal broth with catabolite repression
SSB	Single stranded binding
ssDNA	Single stranded DNA
T	Thymidine
T	Threonine
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
Topbp1	DNA topoisomerase 2-binding protein 1-A
TopoII	Topoisomerase II
TRAIP	TRAF interacting protein
UBA-UBX	Ubiquitin-associated and ubiquitin-regulatory X
Ubi	Ubiquitin
Ubm	Ubiquitylation dead M-phase
Ubs	Ubiquitylation dead S-phase
Ubxn7	UBX domain protein 7
Ufd1	Ubiquitin Recognition Factor 1
UPS	Ubiquitin-Proteasome System
Usp5	Ubiquitin carboxyl-terminal hydrolase
UV	Ultraviolet
V	Volts
VCP	Valosin-containing protein
WRN or FFA-1	Werner

Wt	Wild type
xlZPC	Zona pellucida C glycoprotein
Zp2	Zona pellucida sperm-binding protein
Zp4	Lzpb-a protein

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