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The Role of UFMylation in Fanconi Anaemia

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“The Role of UFMylation in Fanconi Anaemia”

Master of Science by Research
Thesis

School of Medical and Health Science

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

I confirm that I am submitting this work with the agreement of my Supervisor.

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Abbreviations

AP	Apurinic/Apyramidic site
ATP	Adenosine Triphosphate
BRCA1	Breast cancer type 1 susceptibility protein
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DSMO	Dimethylsulfoxide
ECL	Enhanced Chemiluminescence Western Blotting Substrate
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
EVI1	Ecotropic viral integration site 1
FA	Fanconi Anaemia
FBS	Foetal Bovine Serum
HEK	Human Embryonic Kidney
HR	Homologous Recombination
ICL	Inter-strand cross link
ID2 complex	FancI/FancD2 complex
IP	ImmunoPrecipitation
kDa	Kilo Dalton
MMC	Mitomycin C
MRN	MRE11-Rad50-NSB1 complex
PANC or PaCa	Pancreatic Cancer
PBS	Phosphate buffered saline
PTM	Post translational Modification
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic Acid
RPL26	60S ribosomal protein L26
TBS	Tris-buffered saline
Ub	Ubiquitin
UBA5	Ubiquitin-like Modifier activating enzyme 5
UFC1	UFM1-conjugating Enzyme 1
UFL1	UFM1-specific ligase 1
UFM1	Ubiqutin-fold modifier 1
WB	Western Blot

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Abstract

The regulation of cellular activity by intracellular proteins often involves post translational modifications (PTM) with small charged groups, such as PO_4^{3-}, or small proteins, such as ubiquitin. This thesis explores the role of PTM of proteins with the 9.1kDa protein ubiquitin-fold modifier 1 (UFM1) in a process called UFMylation, within Fanconi Anaemia (FA) cell lines. This follows preliminary data produced by Pierce *et al.*, (Unpublished) which suggested that UFMylation activity of the target protein RPL26, takes place more predominantly in FA mutant cell lines compared to non-FA cell lines.

UFM1 is added to a protein in a similar addition cascade to ubiquitin with a non-Ubiquitin (non-canonical) E1 activating step, E2 conjugating step and E3 ligating step (UBA5, UFC1 and UFL1 respectively). Where Ubiquitination has been extensively identified throughout the cell to upregulate protein production or mark proteins for degradation, UFMylation has a limited set of targets though their roles are widespread. UFMylation has been credited to be involved in endoplasmic reticulum-homology (with RPL26), homologous recombination (with MRE11) and protein regulation (with DDGRK1) amongst other things. The contributing role that the addition of UFM1 has on these targets remains unknown.

FA is a rare autosomal recessive disease characterised by biallelic mutation of any gene within the Fanconi pathway which is crucially is involved with the repair of inter-strand crosslinked (ICL) deoxyribonucleic acid (DNA). Failure in ICL repair, particularly in haematopoietic stem cells, leads to bone marrow failure, haematological defects and increase leukemic risk.

Using western blot analysis to explore UFMylation activity we demonstrated UFMylation activity with RPL26, and the presence of UFMylation cascade proteins, across both FA and non-FA cell lines. Preliminary investigation was commenced to explore the impact of cell flask growth conditions and subsequent cell pellet density on RPL26 UFMylation. The initial study suggested that confluency influenced UFMylation activity; potentially reflecting the cell cycle or cell stress dependent nature of UFMylation activity with RPL26.

Contrary to initial observations, UFMylation of RPL26 is not exclusive to FA cell lines, suggesting a broader regulatory role. We speculate UFMylation's involvement in non-catalytic signal transduction pathways and propose further exploration into UFMylation's cellular responses to stress and its implications for protein regulation.

Keywords:

Fanconi Anaemia, UFM1, UFMylation, Signal Transduction, RPL26, RPL26-UFM1, Western Blot, Immunoprecipitation

1. Introduction

1.1 Fanconi Anaemia

Fanconi anaemia (FA) is a rare autosomal recessive condition that affects approximately 1 in 130,000 individuals worldwide per year (Steinberg-Shemer *et al.*, 2020). Originally characterised in 1927 by Swiss paediatrician, Guido Fanconi, who observed a set of three brothers who exhibited severe aplastic anaemia as well as physical and neurological abnormalities (Moreno *et al.*, 2021; Stivaros *et al.*, 2015). FA diagnosis has since been attributed to biallelic mutation of any of the 23 associated genes within the Fanconi pathway (Moreno *et al.*, 2021).

Biallelic mutation of any FA gene (*Fanc*) is leads to malformation of haematopoietic myeloid and lymphoid stem cells due to the role of FA proteins in DNA damage repair (Kelaidi *et al.*, 2019; Sousa *et al.*, 2016; Suzuki *et al.*, 2015). Haematopoietic stem cell disruption leads to pancytopenia, a deficiency of all blood products (Suzuki *et al.,* 2015). Reduced erythrocytes levels characterise the condition as an anaemia, though the erythrocytes that are successfully differentiated are often macrocytic due to overall bone marrow and oxidative stress occurring (Sousa *et al.*, 2016). Impaired lymphoid differentiation results in neutropenia, leading individuals with FA to be at risk of frequent infections (Aliberti *et al.*, 2009). FA patients experience thrombocytopenia due to FA mutations leading to the inhibition of proplatelet protrusion development within megakaryocytes, Figure 1a, a highly specialised precursor cell responsible for the production of platelets within the bone marrow (Machlus & Italiano, 2019; Patel, Hartwig & Italiano, 2005). Thrombocytopenia also contributes to increased risk of infection due to the thrombocytes role in adaptive immune responses and as clotting factors (Holinstat, 2017; Periayah, Halim & Saad, 2017; Ali, Wuescher & Worth, 2015).

The inactivation of FA genes during foetal gestation can lead to developmental defects of the thumbs and forearms, shown in Figure 1b (Thakur & Hiwale, 2023; Rodríguez *et al.*, 2022). Embryological growth defects within FA can also result in neurological delay and abnormalities of important organs including the heart, kidneys, gastrointestinal tract, and eyes (Mehta & Ebens, 2021; Sathyanarayana *et al.*, 2018; Petryk *et* al., 2015; Habib *et al.*, 2000). Clinical features of FA are associated with a short stature, fertility issues and endocrine insufficiency (Tsui & Crismani, 2019; Castilla-Cortazar *et al.*, 2017). Haematological manifestations associated with FA

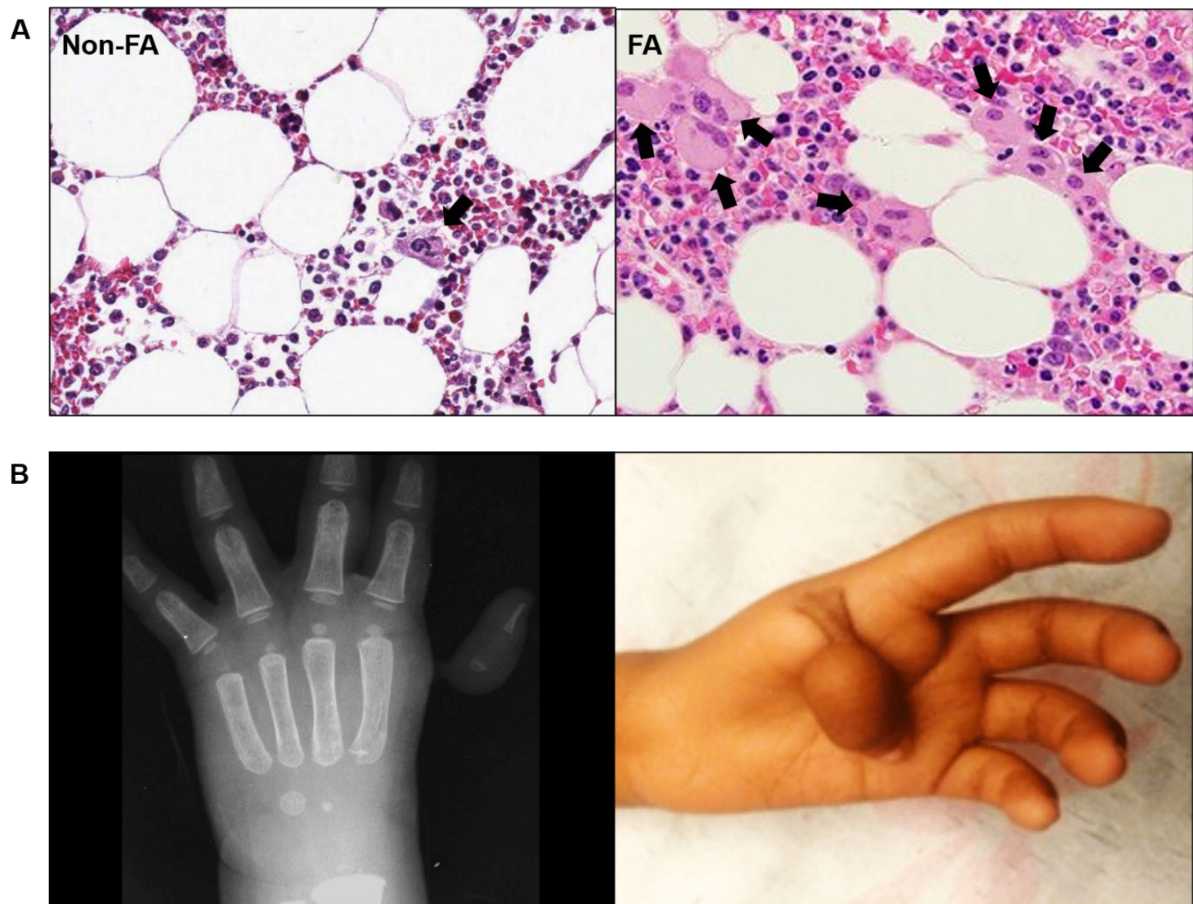

*Figure 1 – Fanconi Anaemia Phenotypes **A** Comparison of 200x haematoxylin and eosin-stained bone marrow tissue in Non-Fanconi Anaemia (FA) (left) (adapted from The Human Protein Atlas, (2023) megakaryocyte detailed by black arrow) and FA (right) (adapted from Kallen, Dulau-Florea & Calvo, (2018) from a 10-year-old with FancJ mutation with atypical megakaryocyte clustering (black arrows). **B** Malformed radial radius of 8-year-old with FancA mutation: Radiograph (left) and photograph (right) adapted from Thakur & Hiwale (2023).*

present in up to 80% of individuals before the age of 10. Severe bone marrow abnormalities that can result in bone marrow failure usually occurs by the age of 8 (Kelaidi *et al*., 2019; Ceccaldi *et al*., 2012)

FA genes encode a series of proteins associated with specialised DNA repair of inter-strand cross links (ICL) (Knipscheer *et al*., 2009). When disrupted as in FA, in addition to chromosome mis-segregation events associated with FA, increasing levels of DNA damage and mutation occur and manifests as a 20% higher likelihood of developing haematological malignancies such as acute myeloid leukaemia (AML) (Alter, 2014; Mehta *et al*, 2007). Solid tumours such as squamous cell carcinoma of the head and neck have been associated to have an earlier onset of up to 30-years in FA patients (Steinberg-Shemer *et* al., 2020; Alter 2014; De Rocco *et al*., 2014; Velleuer and Dietrich, 2014). Expression of ectropic viral integration site 1 (EVI1) has been identified

to increase in bone marrow cells during FA-AML associated malignancy (Masamoto *et al*., 2023; Birdwell Birdwell *et al.,* 2021; Yuan *et al*., 2015). The zinc-finger transcription factor, EVI1, causes a biphasic inhibition of cell cycle progression and differentiation in haematopoietic progenitor cells in FA and non-FA derived myeloid and epithelial malignancies (Birdwell *et al*., 2021; Kustikova *et al*., 2012).

1.1.1 FA Management

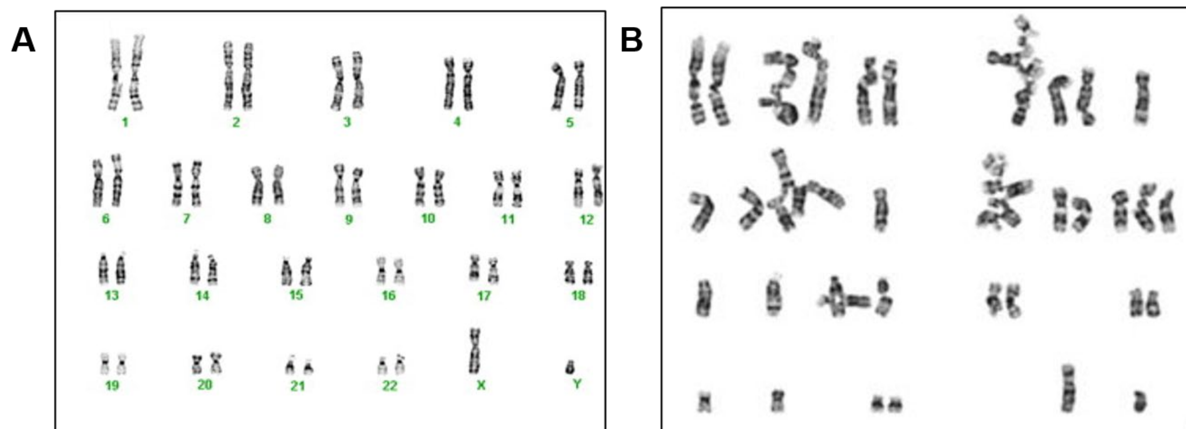

Figure 2 – Karyotype spread ***A*** *Adapted from Wang et al (2009) demonstrating a normal chromosome karyotype spread* ***B*** *Adapted from Lee et al, (2012). 4-year-old with multiple FancA mutations diagnosed with Fanconi Anaemia prenatally karyotype spread following lymphocyte exposure to MMC. Child developed short stature, skin discolouration and disformed fingers, his blood count suggested persistent thrombocytopenia, common in FA.*

FA is usually diagnosed in young children following identification of the high-risk abnormalities previously mentioned. 10% of FA individuals are diagnosed in young adulthood following investigation of leukaemia or solid tumours (Bhandari, Pawan & Puckett, 2022). Diagnosis can be achieved through blood composition testing (macrocytosis and cytopenia), and cytogenetic chromosome breakage tests run on peripheral blood samples from the patient Figure 2 (Lee *et al*., 2012; Oostra *et al*., 2012). Once diagnosed, individuals with severe haematological dysregulation can be treated with blood transfusions and androgen therapy with oxymetholone to stimulate haematopoietic stem cell proliferation. Some patients undergo bone marrow transplantation from a human leukocyte antigen (HLA)-matched donor, often a family relation (Mitchell *et al*., 2014). However, as FA is an inherited condition the bone marrow donor would also need to under-go genetic testing (Oostra *et al*., 2012). Up to 75% of patients experience a permanent curing of the haematological complications associated with FA following bone marrow transplantation (Dufour & Pierri, 2022).

FA cell lines have an increased sensitivity to the DNA damaging agent Mitomycin C (MMC), due to the cells disrupted inter-strand crosslink (ICL) repair pathway (Merfort *et al*., 2022). Figure 2 demonstrates a karyotype spread from an individual with FA who had been diagnosed with FA following chromosome breakage test with MMC (Lee *et al*., 2012). Chromosome breakage testing utilises the inter-strand crosslinking agent MMC to induce ICLs through the addition of a N^2-guanine residue that links the two DNA strands and assess the DNA repair capabilities of the individual with suspected FA (Francies *et al*., 2018; Ebens *et al*., 2017). The N^2-guanine residue from MMC is added to an area of DNA lacking nucleotides: an apurinic/apyrimidinic (AP) site (Hammarsten *et al*., 2021; Housh, *et al*., 2021).

1.1.2 FA associated ICL repair pathway

ICLs can form at AP sites between DNA strands leading to replication fork stalling. Individuals with FA are extremely susceptible to the DNA damaging effect of platinum compounds including the chemotherapeutic cisplatin as well as other chemotherapies containing chloroethylamine functional groups (Ghosal *et al.,* 2020; Lopez-Martinez, Liang & Cohn, 2016; Jabbour, Salem and Sidell, 2014). Exogenous environmental exposures including pollutant carcinogens in cigarette smoke and exhaust fumes, alcohol metabolites and acetaldehyde including dietary fats can also lead to ICL formation (Clauson *et al*., 2013; Langevin *et al*., 2011). Illustrated in Figure 3, stalled DNA replication forks surrounding an ICL are recognised by the FANCM-MHF1-FAAP24 complex (Wienk *et al*., 2013; Tao *et al*., 2012; Singh *et al*., 2010). Hyperphosphorylation of FANCM and FAAP24 via ATM-Rad3-related (ATR) cascade signals the check point kinase ChK1 for checkpoint signalling (Collis et al., 2008). The checkpoint signalling recruits the FA Core Complex composed of modular groups: FANCE, FANCF & FANCC; FANCG, FANCA with FAAP20 and FANCB, FAAP100 and FANCL (a ubiquitin E3 ligase) (Tan & Deans, 2021; Knipscheer *et al*., 2009). FAAP20 is subjected to acetylation on Lysine 152 to protect from proteasomal-degradation signalling by ubiquitination (Nagareddy, Khan and Kim, 2020). This would lead to the polySUMOylation and ubiquitination of FANCA, the degradation of FANCA and therefore failure to compile the FA core complex (Nagareddy, Khan & Kim, 2020; Miteva *et al*., 2013).

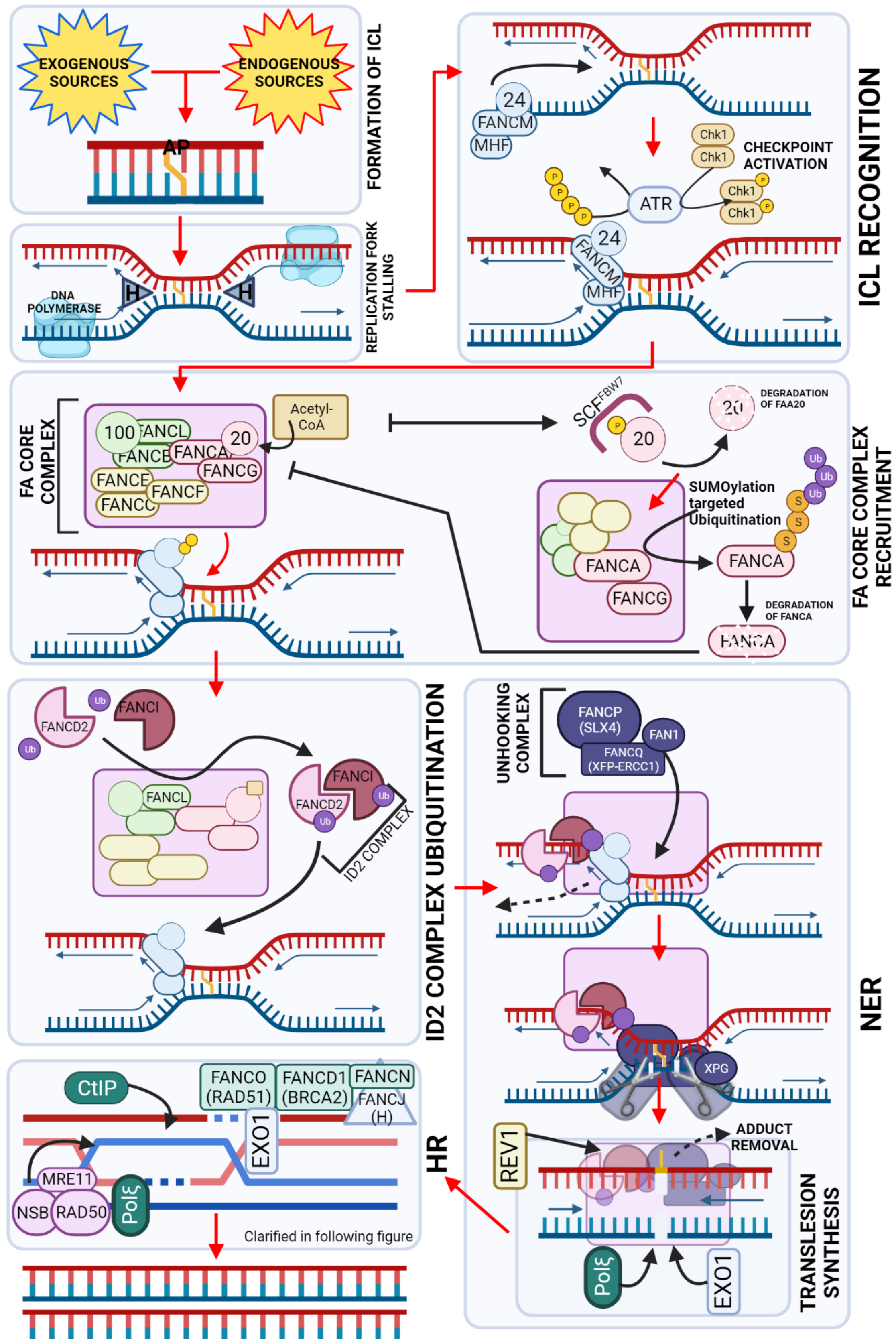

*Figure 3 – **ICL repair by the FA pathway.** Created with BioRender.com illustration depicting ICL repair by the FA pathway. ICLs form at an AP site and cause DNA replication fork stalling. The stalling is recognised by the FANCM-FAAP24-MHF1/2 complex which establishes a cell cycle checkpoint. Cell cycle check point activation recruits the FA Core Complex, composed of FANC proteins detailed, to surround the stalled*

DNA. The acetylation of FAAP20 is essential for the protection of FANCA and the recruitment of the FA Core Complex. The ID2 complex is recruited following the ubiquitination of the components by FANCL. ID2 involvement recruits the unhooking complex utilised to create a double strand break stabilised by EXO1, REV1 and Pol ξ. HR repair pathways also involve FANC proteins, continued in Figure 4.

FANCL is a ubiquitin E3 ligase that recruits and ubiquitinates the ID2 complex (FANCD2/FANCI) (Xie *et al*., 2015). ICL is unhooked by FANCP (SLX4) and FANCQ (XFP-ERCC1) and FAN1 for stability, recruited by Ub-ID2 complex (Imani Nejad *et al*., 2020; Klein Douwel *et al*., 2014; Cybulski & Howlett, 2011). The resulting single strands of DNA, one with a single strand DNA break repaired with nucleoside excision repair REV1 and Pol ξ and the other with a nucleotide adduct that is removed with NER machinery then repaired and replication is completed with the HR machinery FANCO (Rad51); FANCD1 (BRCA2); FANCJ and FANCN as well as the MRN complex (MRE11; NSB1; RAD50), with CtIP and EXO1 for stability of the strand invasion, illustrated in Figure 4.

Upon successful formation of the FA core complex, the ubiquitin ligase FANCL (UBE2T) will monoubiquitinate FANCI and FANCD2 (ID2 complex) (Knipscheer et al, 2009). This reversible post translational modification of FANCD2 is crucial for the recruitment of the downstream effector complex (Kottemann & Smogorzewska, 2016), consisting of FANCQ (XPF-ERCC4) and FANCP (SLX4) with FAN1 to stabilise the ICL site and encourage the nucleolytic incision through a process called unhooking (Imani Nejad *et al.*, 2020; Tan *et al.,* 2020; Klein Douwel *et al*., 2014).

The FANCQ heterodimer mediates repair of DNA lesions with bulky adducts such as ICLs, it cleaves a ssDNA 3’ break on the 5’ side of the ICL and its supporting XPG cleaves on the 3’ side of the damage (Faridounnia, Folkers & Boelens, 2018). Upon this cleavage a 3’-OH group is formed to aid DNA strand invasion during HR. The two DNA strands are then separated, though one retains the nucleolytic lesion. FANCP’s

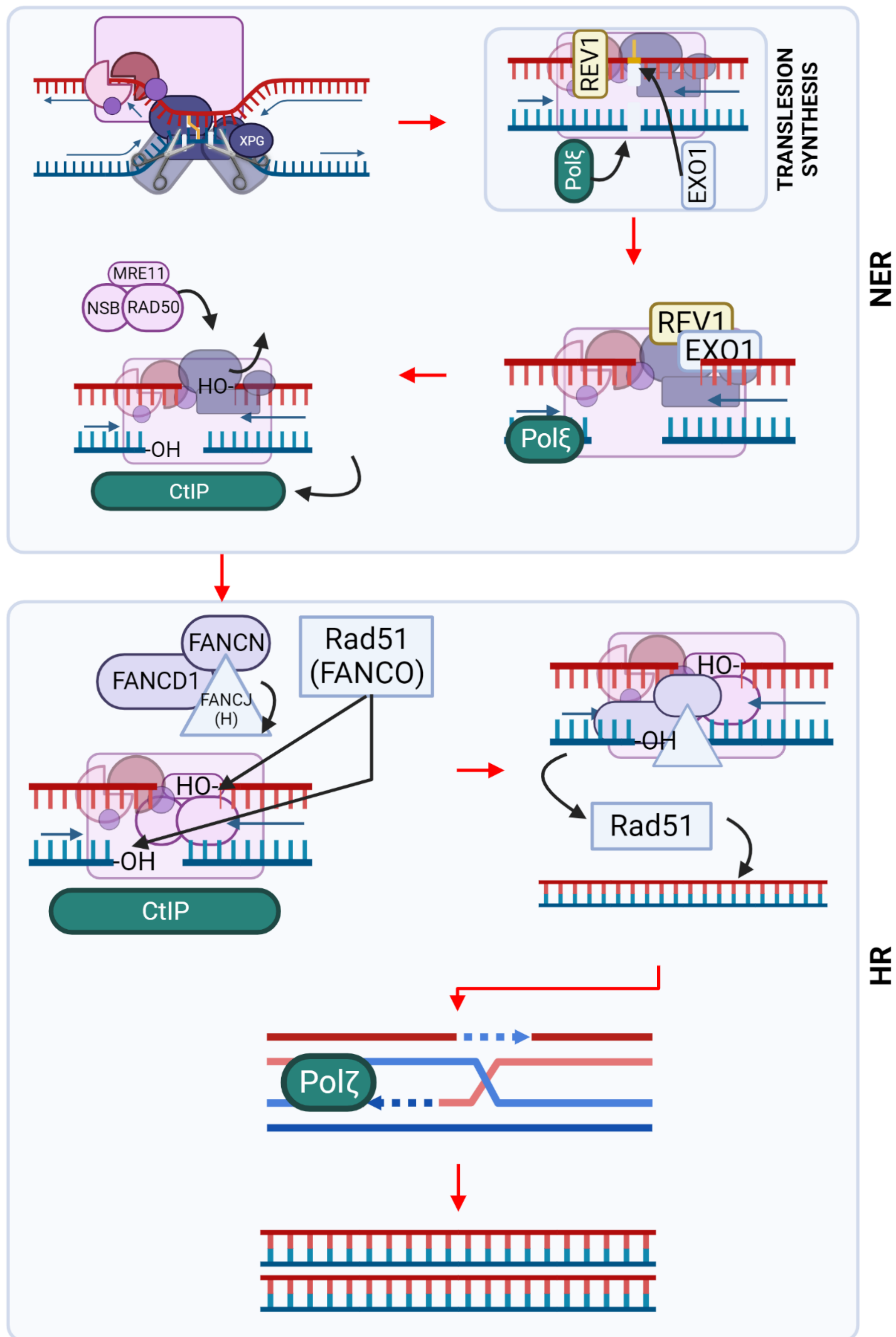

Figure 4 - ***NER and HR associated with FA DNA damage repair.*** *Created with BioRender.com illustration depicting ICL repair by the FA pathway. NER repair of an ICL continues following the FANCP (SLX4) and FANCQ (XFP-ERCC1) and FAN1 unhooking the ICL. The exposed DNA ends are resected 3' to 5' Polξ and 5' to 3' by EXO1 creating a double strand break recruiting HR repair pathway. Check point established by CtIP and the MRE11-RAD50-NSB1 (MRN complex). The FANC HR proteins are recruited with FANCD1 with FANCN and the FANCJ DNA helicase and subsequently recruit Rad51. Rad51 forms filaments for homologue DNA strand invasion for complementary strand synthesis completing DNA repair.*

ubiquitin binding zinc finger interacts with ubiquitinated FANCD2 for efficient recruitment of nucleases for nucleoside excision repair (Yamamoto *et al*., 2011).

NER completes DNA damage repair (DDR) using the Polξ holoenzyme (FANCV/REV7), expanded upon in Figure 4. Polξ is composed of four-subunits that completes strand resection 25-30 nucleotides from the 3'-OH to create a ssDNA overhang with its 3'-5' exonuclease activity (Deshpande *et al.,* 2023; Henninger & Pursell, 2014). Exonuclease 1 (EXO1) works synergistically with Polξ to accomplish 5' to 3' end resection of the complementary DNA strand to create a double strand break (Morafraile *et al*., 2020; Chen *et al*., 2017). EXO1 also ensures that the ssDNA resections are stable during the following homologous recombination (HR) and that transcription activity occurs only between the complementary sequences. Polξ can also conduct 5'-3' DNA Polymerase activity, that then transcribes the complementary sequence of the DNA excluding the ICL (Deshpande *et al*., 2015). REV1 ensures that the cross-linked nucleotides are bypassed on the complementary DNA strand (Budzowska *et al*., 2015; Sharma & Canman, 2012).

The C-terminal interacting protein (CtIP), a co-factor to the MRE11-RAD50-NSB1 (MRN) complex, establishes a cell cycle check point continuing HR (You & Bailis, 2010; Huertas & Jackson, 2009). DNA end resection and stabilisation by FANCQ heterodimer is augmented by the MRN complex (Zhao *et al*., 2020). FANCN (PALB2) interacts with FANCD1 (BRCA2) as a tumour suppressing partner to localise to DNA damage repair (Holloman, 2011). FANCD1 with FANCN and the FANCJ (BACH1/BRIP1) DNA helicase, are required for the formation of Rad51 filaments with the 3'-OH group on the resected DNA strand for DNA homologue searching and DNA strand exchange during HR (Renaudin & Rosselli, 2020; Kais *et al*., 2016). FANCJ is utilised to prevent large deletions near guanine dense quadruplex DNA motifs limiting resection (London *et* al., 2008). Once a DNA homologue is found, Rad51 invades the DNA strand and utilises DNA polymerase ζ (Polζ) activity to transcribe a complementary sequence for the over hangs, excluding the ICL (Budzowska *et al*., 2015; Sharma & Canman, 2012).

Up to 90% of FA diagnoses are caused by mutations within the *FancA*, *FancC* or *FancG* genes (Jeong *et al*., 2020). Though mutation within any module of the core complex can result in the failure to assemble and the disruption of the FA pathway

leading to DNA damage repair pathway inhibition and the diagnosis of FA (Rageul & Kim, 2020; Lopez-Martinez, Liang & Cohn, 2016). Due to the increased presence of DNA damage, cells are more prone to the accumulation of harmful mutations that can lead to cancers including AML (Lavallée *et al*., 2015; Lugthart *et al*., 2008).

1.2 Post translational modification

Post-translational modifications (PTMs) of proteins facilitate the regulation of cellular activity, and the localisation and specificity of interactions (Ramazi & Zahiri, 2021). PTMs diversify the functionality of a protein, often in a reversible fashion, and stimulate specific active sites (Duan & Walther, 2015). PTMs influence gene expression, activation, and DNA repair mechanisms, as illustrated above.

Over 400 distinct types of PTMs have been identified but 24 major PTMs are recognised most widely. These include the addition of acetyl, phosphoryl, glycosyl and methyl groups. SUMOylation, phosphorylation and ubiquitination in particular have been associated with the widest amount of pathway applications.

1.2.1 SUMOylation

SUMOylation is a PTM with the covalent attachment of small ubiquitin-related modifier (SUMO) proteins to lysine residues on target proteins. It has been associated with regulation of genomic stability, cell proliferation and inhibitory roles (Celen & Sahin, 2020). Within the FA pathway the SUMOylation and ubiquitination of FANCA can result in degradation and collapse of ICL repair (Coleman & Huang, 2016). The heterodimer formation of FANCA with FAAP20 is essential for the recruitment of the ID2 complex. FAAP20 protects FANCA from proteasomal degradation by directly interacting with FANCA's SUMO-targeted degron. If exposed FANCA is subjected to SUMO-targeted ubiquitination by RNF4 leading to its own degradation (Nagareddy, Khan & Kim, 2020).

1.2.2 Phosphorylation

Phosphorylation is the addition of a phosphoryl (PO_3) group via adenosine triphosphate (ATP) hydrolysis with a kinase onto a protein (Ardito *et al*., 2017). Phosphorylation acts as a regulator of signal transduction and can target different amino acids within a protein depending on the intended purpose of the PTM (Ardito *et al*, 2017). Phosphorylation of tyrosine is extremely important in pathway signalling whereas phosphorylated serine is widely observed during tumorigenesis resulting in the downregulation of DNA damage repair (Padilla-Mendoza *et al*., 2020).

Serine phosphorylation takes place at hundreds of points within the FA pathway. One such example includes the poly-PO_4^{3-} addition to Ser317 of ChK1 in FAAP24 to aid FANCM-MHF1/2, which is phosphorylated on Ser1045, binding to the DNA at the stalled replication fork facilitated by ATR which simultaneously phosphorylates CHK1 to signal the stalling (Singh *et al.,* 2013*;* Wang *et al*., 2013; Huang *et al*., 2010). Phosphorylation also takes place in the HR pathway with the PO_4^{3-} addition to MRE11 at Ser104 and Ser676 as a pivotal point of resection in the preparation of the DSB ends (Kijas *et al*., 2015). This then influences the phosphorylation of EXO1 leading to deregulation of 5' to 3' strand resection (Tomimatsu *et al*., 2014).

1.2.3 Ubiquitination

Ubiquitination is the addition of small (8.5kDa) protein ubiquitin to ubiquitin-binding domains on proteins (Seyfried *et al*., 2008). Through extensive research conducted since its discovery in 1975, the binding domains can include Lysine, Cystine, Serine and threonine. Ubiquitin binds to lysine via an isopeptide bond, cystine via thioester bond, and binds to serine and threonine via ester bonds (Callis, 2014; Hurley *et al*., 2006). Ubiquitin binds directly to its E cascade proteins N-terminus via a peptide bond (Callis, 2014; Scaglione *et al*., 2013). Ubiquitin has been identified in almost every cellular pathway with its role in maintaining protein homeostasis (Luo *et al*., 2016). This involves activation of tertiary structure proteins to catalyse subsequent protein-protein interactions in addition to ubiquitin's association with the proteasome system to mark proteins for degradation (Bhattacharyya *et al*., 2014).

Ubiquitin is essential for the formation of the ID2 complex within the FA pathway to conduct ICL repair. Though ubiquitin is not the only PTM that acts upon this pathway, it is a crucial step. Transferring onto a protein via a three-step enzyme cascade, the pathway for ubiquitin addition follows an E1, E2, and E3 cascade: Ub-activating enzyme; Ub-carrier enzyme; Ub-ligase enzyme respectively (Damgaard, 2021). There are many E1, E2 and E3 suitable for ubiquitination and are referred to in literature as canonical due to their similar domain structures and enzymatic activity, though non-canonical steps are accepted for alternative PTMs (Stewart *et al*., 2016; Callis, 2014; Hurley, Lee & Prag, 2006).

1.2.4 UFMylation

New PTMs are continually being discovered, though their definitive targets and functions within protein activity are mostly unknown.

UFMylation, associated with the ubiquitin-fold modifier 1 (UFM1) protein was discovered in 2004 by Komatsu *et al.* Its small size, non-enzymatic properties and similar tertiary structure to ubiquitin, Figure 5a, classes UFM1 as a ubiquitin-like protein (Banerjee, Kumar & Wiener, 2020; Merbl *et al*., 2013; Sung *et al*., 2007). Though bigger than ubiquitin at 85 amino acids and an atomic mass of 9.1kDa, UFM1 only shares 16% of its protein code with ubiquitin (Banerjee, Kumar & Wiener, 2020). UFM1 also shares the characteristic glycine residue at its C-terminus followed by a serine-cystine dipeptide that is used for activation of a ubiquitin-like protein (Streich & Lima, 2014). UFM1 covalently attaches to its target proteins via an isopeptide bond to lysine residues of substrate proteins in a similar cascade fashion as ubiquitin, Figure 5b (Banerjee, Kumar & Wiener, 2020; Komatsu *et al*., 2004).

UFMylation adds UFM1 proteins in a similar cascade manner as ubiquitination with non-canonical E complexes, Figure 5b (Peter *et al*, 2022; Banerjee, Kumar, and Wiener, 2020). UFM1 is initially prepared into a pro-form with the cleaving of a serine-cysteine dipeptide at its C-terminal by UFM1 specific proteases (UFSP) 1 (UFSP2 in humans) (Millrine *et al.,* 2022). A then mature UFM1 interacts with non-canonical E1 ubiquitin-like modifier activating enzyme 5 (UBA5) via a non-covalent complex with ATP conjugation at the adenylation domain cystine 250, activating UFM1 in a similar fashion to Ub-E1 (Kumar *et al*., 2021; Gavin *et al*., 2014). UFM1-conjugating enzyme (UFC1) acts as E2 and interacts with the ubiquitin-fold domain of UBA5 to transfer UFM1 to the active site of UBA5, accepting UFM1 with a thioester bond with cystine 116 (Kumar *et al*., 2021). UFM1-specific ligase 1 (UFL1) acts as E3 to bind UFM1 at its topoisomerase transcription factor binding site and facilitates substrate transfer. UFL1, as the only identified E3 ligase, has been associated with all UFMylation targeting and has been suggested to act as an Ataxia-Telangiectasia-Mutated (ATM) kinase instigator (Qin *et al*., 2019). ATM kinase is a DNA damage transducer (Menolfi & Zha, 2020).

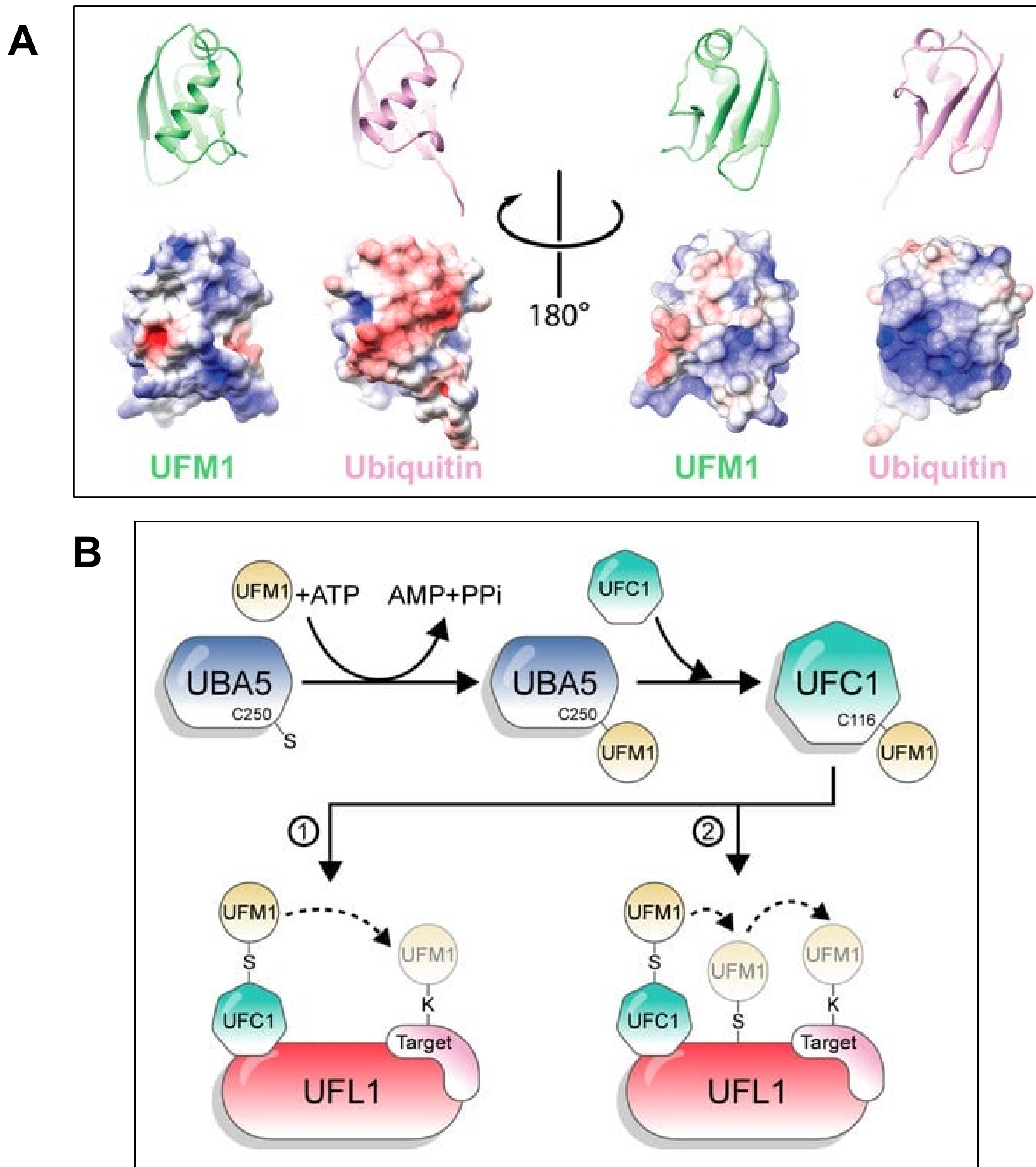

Figure 5 – UFMylation ***A*** *Ubiquitin and UFM1 share similar tertiary structures though only 16% of their protein code is replicable (Protein structure diagram adapted from Banerjee, Kumar & Wiener, 2020)****B*** *The UFMylation cascade with non-canonical E1 (UBA5);E2 (UFC1) and E3 (UFL1) (Pathway illustration by Banerjee, Kumar & Wiener, 2020).*

The full understanding of the role of UFMylation is limited. UFMylation has been identified as a PTM in relation to a range of intracellular processes though no isolated purpose defines it.

UFM1 has been shown to modify eukaryotic translation initiation factor 4E (eIF4E) in protein translation; Ribosomal Protein L26 (RPL26) and DDRGK domain-containing protein 1 (DDRGK1) in endoplasmic reticulum (ER) homeostasis and MRE11 in DNA damage repair (Scavone *et al.,* 2023; Lu *et al*., 2021; Sung *et al*., 2007).

eIF4E recognises and binds to 5’ messenger ribonucleic acid (RNA) during the initiating of protein synthesis during protein translation (Xu *et al*., 2010). UFMylation of eIF4E has been identified to assemble the eIF4F translation initiation complex which in turn interacts with cycle D1 in cell cycle signalling for cell stress response (Gak *et al*., 2020). UFMylation of MRE11 has been recently recognised as a required process to assemble the MRN complex during ds-DNA break repair in homologous recombination and for the maintenance of telomere length to prevent premature aging of a cell (Lee *et al*., 2021; Wang *et al*., 2019). DDRGK1 is a cytoplasmic protein that has been related to mitochondrial metabolism (Liang *et al*., 2020). DDRGK1 is theorised to facilitate ER-resident UFMylation via the relocation of the non-canonical E3 UFL1 (Liang *et al*., 2020).

UFM1 has also been identified to modify histone H4 in the nucleus of the cell (Qin *et al*., 2019). Histone H4 is essential in the stabilisation and compaction of chromatin within the nucleosome. Histone H4 aids in the stabilisation of replication forks during DNA damage repair to aid synthesis of a correct copy of the genome (Clouaire *et al*., 2018). UFL1 UFMylates Histone H4 at lysine 31 where it then supports Histone H3’s trimethylation to become transcriptionally active at a ds-DNA break (Qin *et al*., 2019; Clouaire *et al*., 2018). This, in turn, leads to the activation of ATM kinase and DNA damage repair. A positive feedback loop is formed due to Histone H4 also phosphorylating UFL1 (Qin *et al*., 2019).

UFMylation has been associated with cellular development and tumour progression in relation to p53 and oestrogen-receptor-α, following CRISPR-depletion research in cell lines highlighting upregulated UFMylation activity (Yoo *et al*., 2022; Liu *et al*., 2020). Murine studies have demonstrated UFM1 as a crucial protein as depleted mutants were not viable, simultaneously the UFMylation pathway protein UFC1 is upregulated during skeletal muscle growth in post-natal development (Molendijk *et al*, 2022).

UFMylation holds an important role throughout cellular development and during intracellular processes but unlike its name’s sake ubiquitin, there is no clear function that defines UFMylation.

Ubiquitination of a protein often highlights it for amplification or marks it for degradation (Hanna *et al*., 2017*;* van Twest *et al*., 2017). UFMylation’s common theme has not yet been identified, though UFM1 seems to commonly instigate a protein pathway. As a

signal transductor UFM1 appears to encourages its UFMylated target activity, more aligned with phosphorylation, though there is no evidence to suggest UFMylation is a catalytic reaction (Ardito *et al.,* 2017; Lee & Yeffe, 2016).

1.2.4.1 RPL26

Ribosomal protein L26 (RPL26), a 17kDa cytoplasmic protein that has several lysine residues embedding it to the ER, is widely accepted as the primary target of UFMylation (Scavone *et al*., 2023; Walczak *et al*., 2019). RPL26 is believed to regulate the translation of tumour suppressor proteins p53 and p73 (a p53 family tumour suppressor) following DNA damage though its exact action is unknown (Scavone *et al*., 2023; Hayashi *et al*., 2019; Zhang *et al*., 2016; Chen, Guo & Kastan, 2012; Takagi *et al*., 2005).

Discovered as the main target during mass spectroscopy analysis of immunoprecipitants prepared with UFM1 antibody, RPL26 is associated with the quality control of proteins produced by the ER (Zhang *et al*., 2012). It is suggested that UFMylated RPL26 instigates ER-phagy as a response to cellular stress and in maintenance of the subsequent protein stalling (arrested peptides) (Scavone *et al*., 2023; Stephani *et al.,* 2020; Wang *et al*., 2020). ER-phagy is the selective break down of ER sheets within the double phospholipid bilayer of a secondary lysosome during times of ER stress – which itself results in the unfolded protein response (Mochida & Nakatogawa, 2022; Yang *et al*., 2021). UFMylated RPL26 facilitates the lysosomal transport of ER-phagy via the association with UFL1 and the UFMylation support protein DDRGK1 (Savone *et al*., 2023; Walczak *et al*., 2019)

A frame shift mutation of the RPL26 coding gene has been associated with the development of the very rare inherited condition, Diamond-Blackfan anaemia (Gazda *et al*., 2012). Diamond-Blackfan anaemia is characterised by macrocytic-normocytic red cell anaemia, congenital bone abnormalities and thumb malformations, similar to FA (Gadhiya & Wills, 2023; Engidaye, Melku & Enawgaw, 2019; Tsilou *et al*., 2010). Differing from FA by only causing a low red blood cell count without impacting other blood components and no later malignant risks, Diamond-Blackfan anaemia only has 7 new cases per 1 million live births per year (Fatima *et al*., 2021; Tsilou *et al*., 2010).

1.3 Rationale

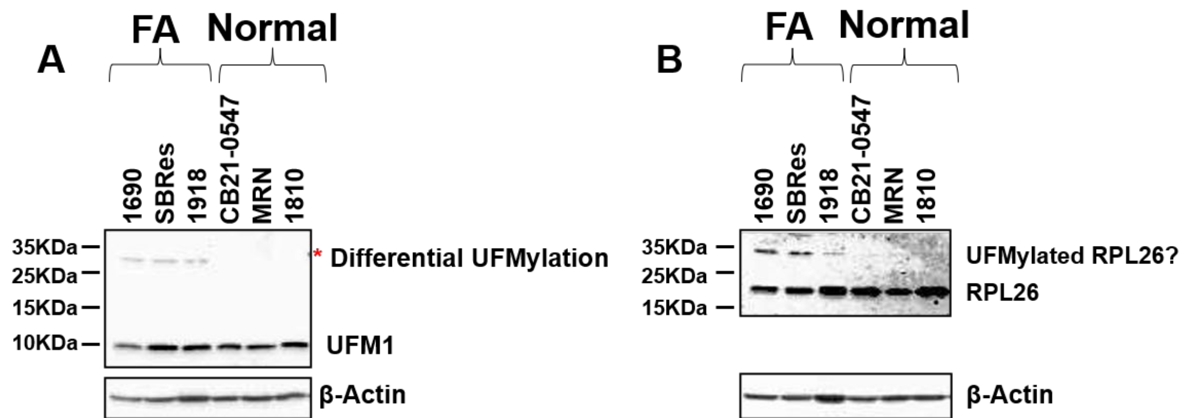

*Figure 6 – **Preliminary Western Blot Data.** Western Blot run by Pierce et al. (Unpublished) with 30ug protein from the labelled cell lines of control and Fanconi Anaemia origin. β-Actin used as loading control. **A** Probed with antibody against UFM1 **B** Probed with antibody against RPL26.*

This project follows the preliminary western blot data produced by Pierce *et al*. (Unpublished) (Figure 6). This data demonstrates that when probed with UFM1 antibody a UFM1 band present at 10kDa as expected but also a secondary protein band is present with approximate molecular weight of 27kDa within only the FA cell lines used and not the control/non-FA cell lines, Figure 6a. From examination of the literature and reported major targets of UFMylation Pierce *et al.,* (Unpublished) theorise this to be UFMylated RPL26 (Wang *et al*., 2020; Walczak *et al*., 2019). A second western blot, Figure 6b, produced with the same cell lines probed with an RPL26 antibody detected a band at 27kDa. This band was again only detected in the FA cell lines suggesting that the band at 27kDa band in the FA cell lines could indeed be UFMylated RPL26.

Preceding this project there has been no direct association between RPL26 and FA or FA and UFMylation. It is recognised, *in vitro* deficiency of RPL26 does cause Diamond-Blackfan anaemia that demonstrates some similar developmental and anaemia symptoms as FA. UFMylation activity has been identified with the HR protein MRE11 (Lu *et al*., 2021; Wang *et al*., 2019). MRE11 is associated with end resection during the FANC repair pathway (You & Bailis, 2010).

Preliminary data from a Mass Spectrometry based screen of EVI interacting proteins conducted by Meyer *et al*., (Unpublished) suggested EVI1 interacts with UFM1.

2. Aims and Objectives

This project aimed to explore UFMylation and associated protein activity in FA cell lines compared to non-FA cell lines. We wanted to highlight UFMylation activity of RPL26 and identify other UFMylation targets within the FA and non-FA cell lines.

1. Confirm differences in UFMylation targets between FA derived and non-FA cell lines.
2. Determine the expression of the components of the UFMylation pathway in FA and non-FA cell lines.
3. Investigate the effect of MMC and irradiation on UFMylation in FA and non-FA cell lines.
4. Confirm whether the AML associated FA progression factor EVI1 is UFMylated.
5. Investigate the effects of UFMylation on EVI1 function.

3. Results

3.1 UFMylation activity in lymphocyte FA and non-FA cell lines

The following investigation utilised a FA cell line with a *FancP/SLX4* gene mutation, and the non-FA control cell line: MRN, used by Pierce *et al*., kindly gifted by Dr Stephan Meyer of Manchester University.

Initial study sought to reproduce the preliminary western blot data by Pierce *et al*., (Unpublished). The data (Figure 6) detailed the presence of an additional antibody binding band at 27kDa in only FA derived lymphocyte cell lines when probed with UFM1 and RPL26 antibodies: suggesting UFMylation of RPL26 takes place at higher levels in FA derived lymphocyte cell lines.

This study conducted western blots prepared with RIPA buffer lysates of the FA (CB15-0387) and non-FA (MRN) cell lines in non-treated, 24-hour treated with MMC or 24-hour treated with MMC's solvent carrier DMSO. The non-treated lysates probed with an UFM1 antibody, in Figure 7a, display a varied expression of the UFM1 protein at 10kDa, and the PTM band at approximately 27kDa between the MRN (left) and CB15-0387 (right) cell lines. The detected levels of UFM1 and the PTM bands did vary between the cell lines, which appeared to be dependent on drug exposure as all lysate samples were loaded with a consistent protein concentration (30ug). It is noted that despite this the protein binding across all lanes probed with UFM1 antibody, was consistently at 27kDa.

Simultaneously explored was the impact of 24-hour exposure to the ICL-inducing drug MMC or its solvent carrier DMSO, on UFM1 expression and UFMylation activity. There was a low detectable expression of the individual protein, UFM1, following 0.02% DMSO treatment within the non-FA cell line, MRN. However, Figure 7a did suggest the antibody was able to detect the conjugated UFMylation activity at 27kDa. Within the FA mutant cell line (CB15-0387) the detectable UFMylated protein was upregulated following exposure to both DMSO alone and MMC, prepared in DMSO. This in turn could support the idea that UFMylation activity is a cell stress response as DMSO is a strong solvent that can induce DNA damage (Dludla *et al.*, 2021). The relative detectable levels of UFMylation activity did not increase following additional exposure to MMC in either the FA or non-FA cell lines.

When western blots with the FA (CB15-0387) and non-FA (MRN) cellular lysates were probed with RPL26 antibody, Figure 7b, only very faint levels of UFMylated RPL26 were observed at 27kDa with an additional antibody binding observed at 90kDa. The antibody binding with the RPL26 antibody was consistent with the UFM1 antibody probed western blots in Figure 7a, strongly suggesting that the PTM activity agrees with the widely accepted primary target of UFMylation, the ER protein, RPL26. Exposure to 20nM MMC did result in lower of the observed levels of RPL26 in the FA mutant cell line (CB15-0387). This result was contrary to the expected detection of UFMylated RPL26 activity.

Analysis progressed to include alternative element of the UFMylation pathway: the proteins UBA5 and UFC1, the E1 activating protein and the E2 carrier protein for UFMylation respectively.

Probing of the FA and non-FA cell lines with an UBA5 antibody, Figure 7c, did not show any additional antibody binding activity and an overall consistent level of expression between the FA cell line and non-FA cell line. There was a slight down regulation of UBA5 following 20nM MMC exposure in the non-FA cell line that was not replicated in the FA cell line.

Probing with UFC1 antibody, Figure 7d, demonstrated a lower relative expression of UFC1 in the non-FA cell line and FA cell line following exposure to 0.02% DMSO. 20nM MMC exposure showed a relative higher expression of UFC1 in the non-FA cell line, this relative increase was not preserved in the FA cell line following the MMC treatment. In both cell lines additional UFC1 antibody binding was present as 29kDa and 39kDa which was not interfered with by drug treatment. This could be the UFMylation E2 interacting with a single unit or doublet of UFM1 to form its dimer structure (Kumar *et al.,* 2021).

The analysis of the lymphocyte non-FA and FA cell lines used here did not replicate the preliminary data by Pierce *et al*. but the expression of UFMylated RPL26 across the cell lines is consistent with wider literature. There were some differences of expression between the cell lines following drug treatment when probed with UBA5 and UFC1 antibodies, with additional binding observed. Though, the binding of the antibodies observed here could suggest that UFMylation is not a pathway that is affected by *Fanc* gene mutation.

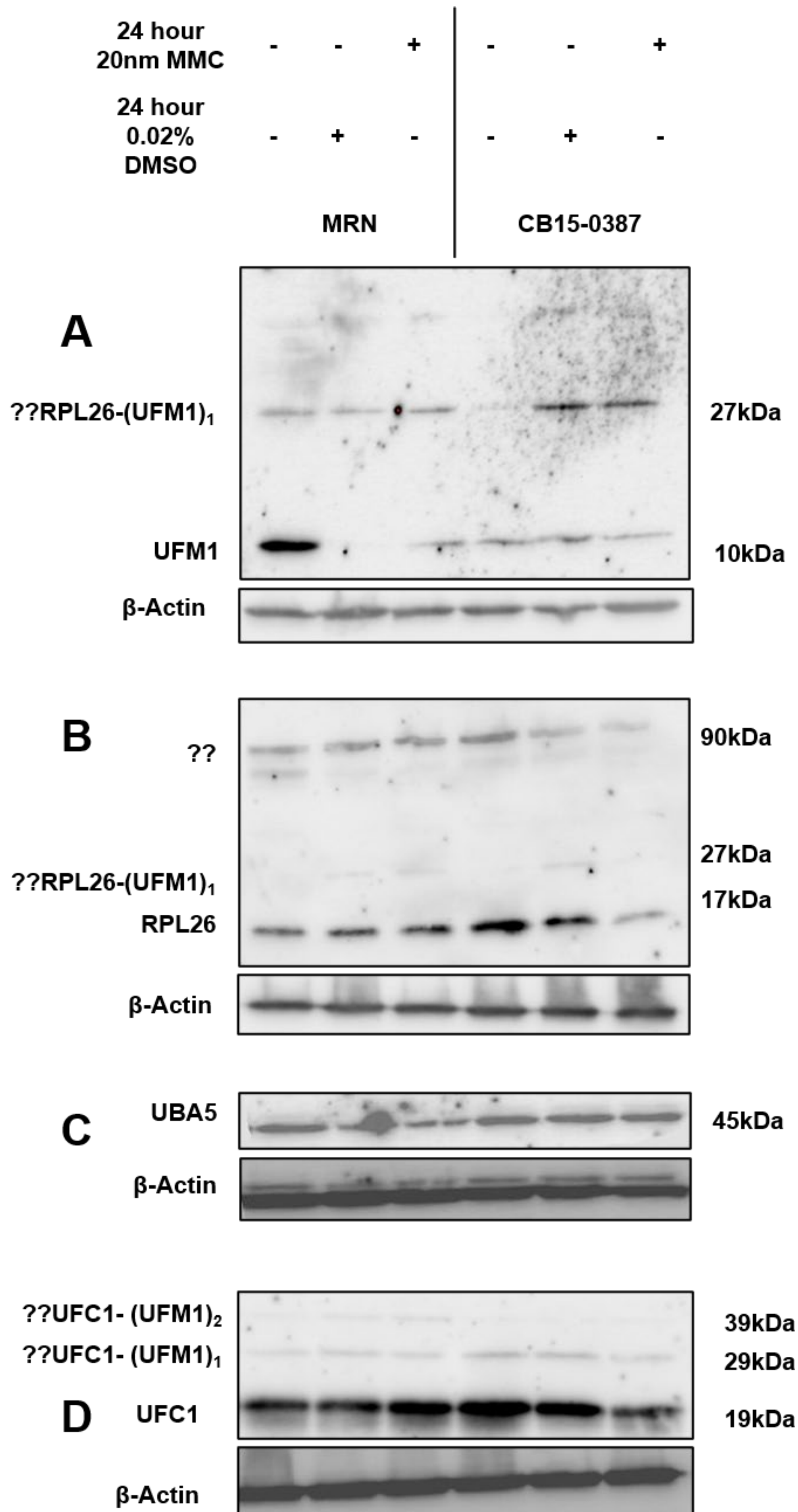

*Figure 7 – **Lymphocyte UFMylation pathway analysis Western Blot.** 12.5% gel with 30µg of two lymphocyte cell lines: MRN (control/non-FA) and CB15-0387 (FancP/SLX4 mutant) in the presence or absence of the drugs detailed. β-Actin used as loading control. **A** Probed with UFM1 antibody. **B** Probed with RPL26 antibody. **C** Probed with UBA5 antibody. **D** Probed with UFC1 antibody.*

3.2 FA cell line, CB15-0387, does not show increased sensitivity to increasing MMC concentration compared to non-FA cell line, MRN.

Following western blot analysis that demonstrated UFMylation activity in both the FA and non-FA cell lines that differed from Pierce *et al*'s preliminary data, a 0-150nM dose sensitivity assay with the ICL inducing drug MMC was conducted, Figure 8a. This explored the viability of the lymphocyte FA and non-FA cell lines. It was predicted that the FA mutations would have an increased sensitivity to MMC exposure compared to non-FA cell lines due to FA's impaired ability to repair ICL associated DNA damage.

Incubated over 72-hours with a 3-hour WST-1 reagent incubation (Supplementary Figure 1) the MMC sensitivity assays demonstrated a generalised sensitivity to increasing MMC dose. Differing from the expected results, the drug dose assays demonstrated no significant difference between the FA cell line (CB15-0387, yellow) and the non-FA cell line (MRN, green) (Figure 8a). The undifferentiable sensitivity between the cell lines following MMC exposure was independent of their growth abilities with in the 96-well plate. Figure 8b details the average absorbance levels of the internal growth control which utilised a serial dilution and was present during every assay. It demonstrates the $5x10^5$ cells/ml seeding density of the assay was within the mid-logarithmic growth required for consistent, healthy cell growth that avoids additional cell stress factors associated with over-confluency (Galluzzi, Yamazaki & Kroemer, 2018; Halliwell, 2003).

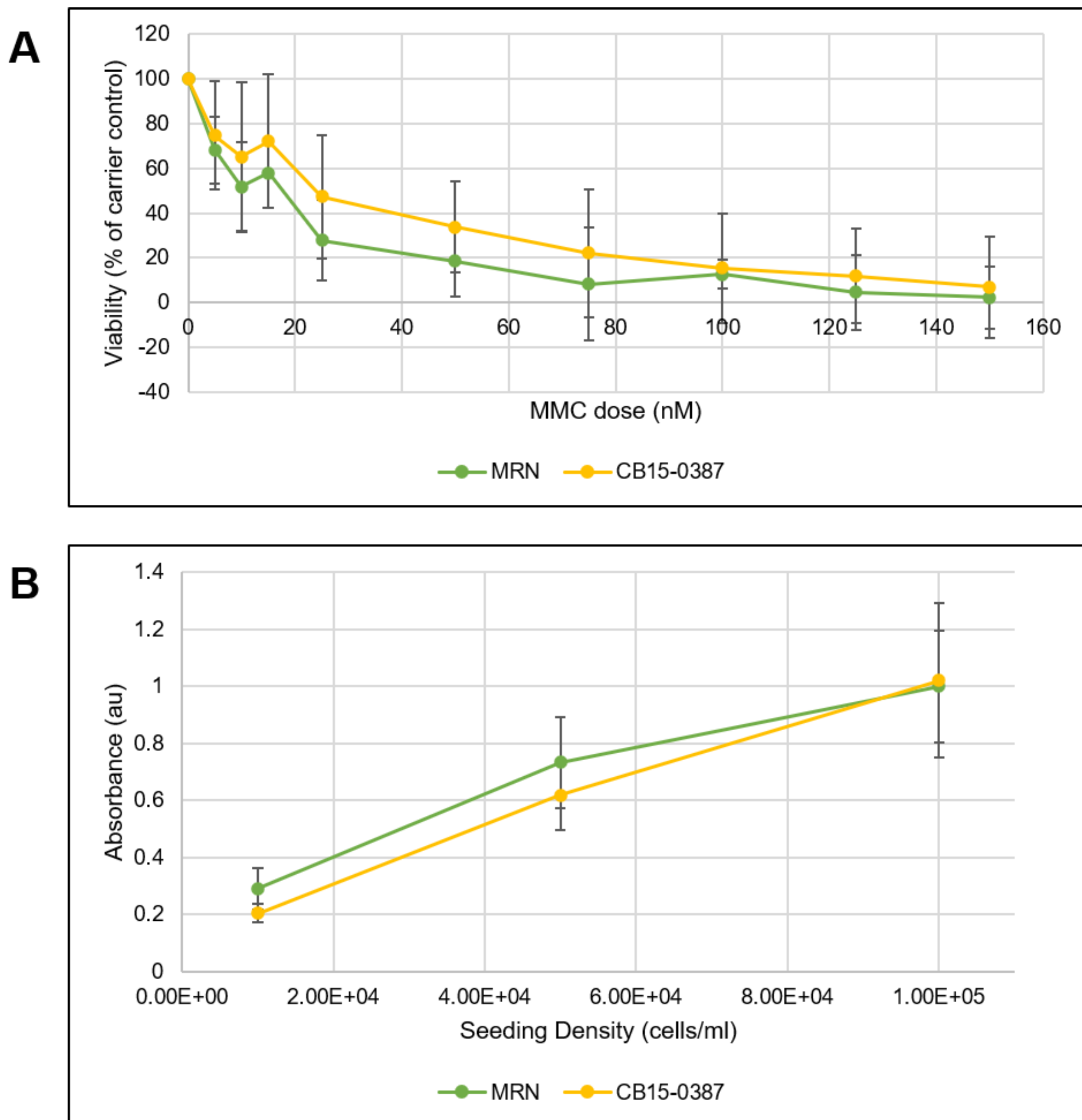

Figure 8 – ***Undifferentiable MMC sensitivity between cell lines.*** *72-hour incubation with 3 hour WST-1 reagent exposure.* ***A*** *Average relative percentage viability (n=5) with increasing MMC treatment (0nM-150nM) of two lymphocyte cell lines (MRN – non-FA; CB15-0387 – FancP/SLX4 mutant) following seeding density of* $5x10^5$*cells/ml.* ***B*** *Average absorbance (n=5) of internal cell growth control (untreated). Error bars for both graphs are representative of standard deviations. Statistical testing with Student T Test demonstrated no statistical significance.*

3.3 *FancD2* Status Queried Integrity of Lymphocyte Cell Lines

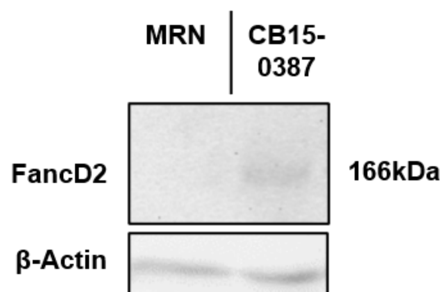

*Figure 9 – **FancD2 Status Queried Integrity of Lymphocyte Cell Line.** 6% Western Blot prepared with 30μg two lymphocyte cell lines: MRN (control/non-FA) and CB15-0387 (FancP/SLX4 mutant) (n=2). Probed with FancD2 antibody, β-Actin used as loading control.*

In response to the undifferentiable MMC sensitivity levels of the lymphocyte FA and non-FA cell lines, the *FancD2* status of the cell lines was assessed. FANCD2 is a crucial step within compilation of the FA pathway for the recruitment of down-stream effector complexes that lead to NER and HR taking place. FANCD2 was expected to be have a strong presence in the MRN control cell line and a slight presence in CB15-0387. This is due to the CB15-0387 cell line's *FANCP/SLX4* mutation. FANCP/SLX4 is the subsequent recruitment step of ID2 complex formation and FANCD2 ubiquitination (Cybulski & Howlett, 2011). FANCP's UBZ interacts with ubiquitinated FANCD2 for efficient recruitment of nucleases for NER (Yamamoto *et al*., 2011). Levels of FANCD2 recruitment has been directly associated with FANCP/SLX4 presence in the assessment of chromosome bridge formation associated with recombination repair suggesting that their recruitment is not mutually exclusive (Sarbajna, Davies and West, 2014).

Figure 9, a western blot probed with FANCD2 antibody, suggested that there was no detectable FANCD2 in the MRN cell line. The expected detection of intact FANCD2 within the CB15-0387 cell line was observed. It is recognised that due to testing the preceding FA pathway step, the mutant status of the CB15-0387 was not explicitly confirmed.

As this point in the investigation, post translational UFMylation activity suggested to be UFMylated RPL26, had been observed in both the MRN and CB15-0387 cell lines with drug dose response assays demonstrating undifferentiable levels of sensitivity to MMC. The divergent data from the preliminary western blot (Pierce *et al*., Unpublished) (Figure 6) and expected MMC sensitivity of an FA cell line, in combination with an

undetectable level of FANCD2 in the MRN cell line, cumulated to suggest that a mutation may have taken place. If a mutation were to have taken place in the MRN cell line it could have caused the cell line to behave as though it contained a FA mutation. It is also acknowledged that the mutant status of the FA cell line was not confirmed and so it is also a possibility that CB15-0387 could have experienced a reversion. There was consideration of contamination of the MRN cell line via human error but retrospective analysis of early western blot data demonstrated UFMylation activity in both the MRN and CB15-0387 (*FancP/SLX4* mutant) cell lines so an earlier passage of the cell lines would not have rectified the issue of FA behaviour.

No other FA or non-FA lymphocyte cell lines utilised by Pierce *et al*., (Unpublished) were available at the time of this project so alternative cell lines were acquired. No further investigation was conducted to explore definitively the mutant status of the lymphocyte cell lines. Investigations were adapted for the use of SV40-transformed FA fibroblast cell line with FancD2 mutant and a retroviral FancD2 transgene PD20 and PD20 RV:D2 respectively (Jakobs *et al*., 1996).

3.4 *FancD2* Status of Fibroblast FA and non-FA Cell Lines Confirmed

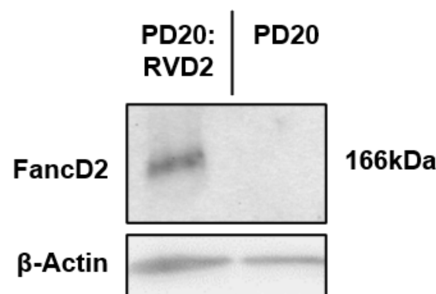

*Figure 10 – **FancD2 Status of Fibroblast FA and Non-FA Cell Lines Confirmed.** 6% Western Blot prepared with 30µg SV40-transformed FA fibroblast cell lines: PD20 (FancD2 $^{-/-}$mutant) and PD20 RV:D2 (FancD2 $^{-/-}$ plus RetroViral FANCD2 transgene). Probed with FancD2 antibody, β-Actin used as loading control.*

Preceding the use of the SV40-transformed FA fibroblast cell lines PD20 (*FancD2* $^{-/-}$) and PD20 RV:D2 (*FancD2* $^{-/-}$ plus RetroViral *FancD2* transgene) for UFMylation investigation, the FANCD2 expression was investigated to confirm its expected status. As reported the PD20 cell line had no detectable presence of FANCD2 and the PD20:RVD2 had a strong FANCD2 expression (Figure 10).

3.5 Significant MMC Sensitivity Difference Between Fibroblast FancD2 mutant and intact cell lines

The MMC sensitivity of the fibroblast cell lines was assessed to demonstrate the sensitivity of the FA mutant cell line to the ICL inducing effects of MMC. The failed

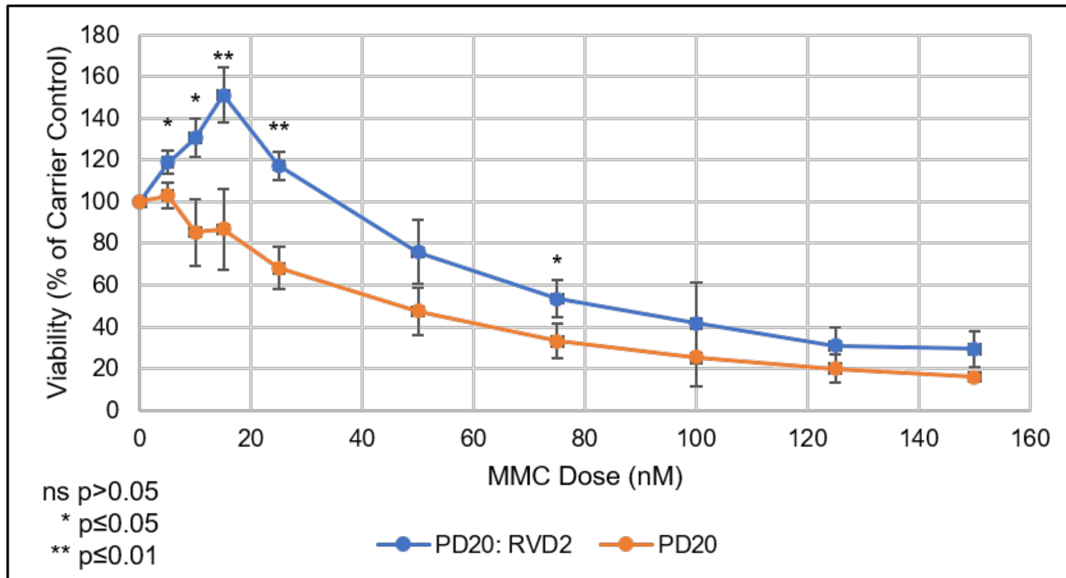

*Figure 11 - **MMC sensitivity of FA cell line A** Average relative percentage viability (n=3) 72 hour incubation with increasing MMC treatment (0nM-150nM) of two fibroblast cell lines (PD20 (FancD2 $^{-/-}$ and PD20 RV:D2 FancD2 $^{-/-}$ plus RetroViral FancD2 transgene) 3-hour WST-1 reagent exposure. Error bars are representative of standard deviations. Statistical significance determined using a Student T-Test.*

induction of ICL-induced DNA repair by the FA pathway due to FANC mutation leads to increased levels of apoptosis following exposure to the drug MMC. The proliferation MMC dose assay for the fibroblast cell lines was seeded at 4×10^4 cells/ml and follow the same conditions as the lymphocyte experiments (Supplementary Figure 2). Assessed using a 0-150nM dose range, the PD20 (*FancD2* mutant, orange) cell line showed significant sensitivity to MMC treatment compared to the PD20 RV:D2 (control, blue) cell line, Figure 11.

3.6 UFMylation Cascade Facilitators in Fibroblast FA

The initial aim of this study was to reproduce the preliminary data produced by Pierce *et al.*, (Unpublished). Following confirmation of their appropriate mutation and MMC sensitivity status, the fibroblasts were used to investigate the UFMylation activity in FA and non-FA cell lines. Figure 12 demonstrates faint evidence of an additional binding presence of UFM1 antibody in both the FA mutant and transgene corrected cell lines (Figure 12a), differing from Pierce *et al*'s data (Figure 6). However, we did not observe any further binding when probed with RPL26 antibody (Figure 12b), inconsistent with both the preliminary data and the lymphocyte studies conducted here. Figure 13 depicts western blots prepared with FA and non-FA fibroblast cell lines in the presence of the ICL inducing 20nM MMC drug or its solvent carrier 0.02% DMSO treatment

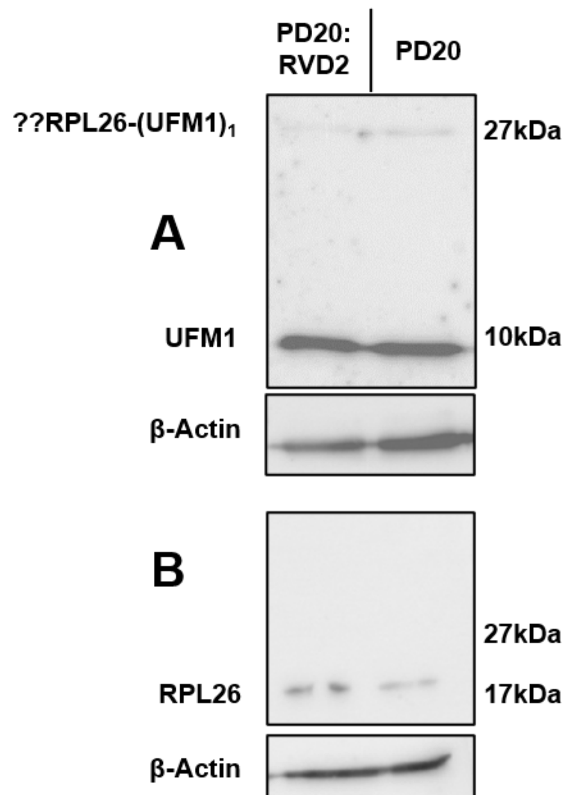

*Figure 12 – **Fibroblast FA UFMylation Activity.** 12.5% Western Blot prepared with 30μg SV40-transformed FA fibroblast cell lines: PD20 (FancD2 $^{-/-}$mutant) and PD20 RV:D2 (FancD2 $^{-/-}$ plus RetroViral FANCD2 transgene. β-Actin used as loading control. **A** Probed with UFM1 antibody. **B** Probed with RPL26 antibody.*

incubated for the time indicated, probed with the UFMylation cascade proteins. When probed with UFM1 antibody, Figure 13a, these lysates suggested that the further binding of the antibody to the PTM of RPL26 was only present in the PD20:RVD2 (*FancD2* $^{-/-}$ plus RetroViral *FancD2* transgene) cell line and scarcely in the PD20

(*FancD2* $^{-/-}$) which differs from Figure 12's untreated samples. This suggests there is variation in UFMylation activity though what this is dependent on is not abundantly clear. When probed with RPL26 there was no detectable presence of a UFMylated band in either cell line regardless of drug treatment, Figure 13b.

Though neither Figure 12b or Figure 13b, both probed with RPL26 antibodies, demonstrated an antibody band at 27kDa to suggest UFMylated RPL26 the activity varied between the cell lines between Figure 12a and Figure 13a. The untreated lysates in Figure 12a demonstrate a 27kDa band in both cell lines, albeit a faint detection, that is not replicated in the FancD2 mutant PD20 cell line in Figure 13a. Practically, the lysate preparation process between the lymphocyte and fibroblast cell lines only differed in the trypsinisation step of the fibroblast cell lines.

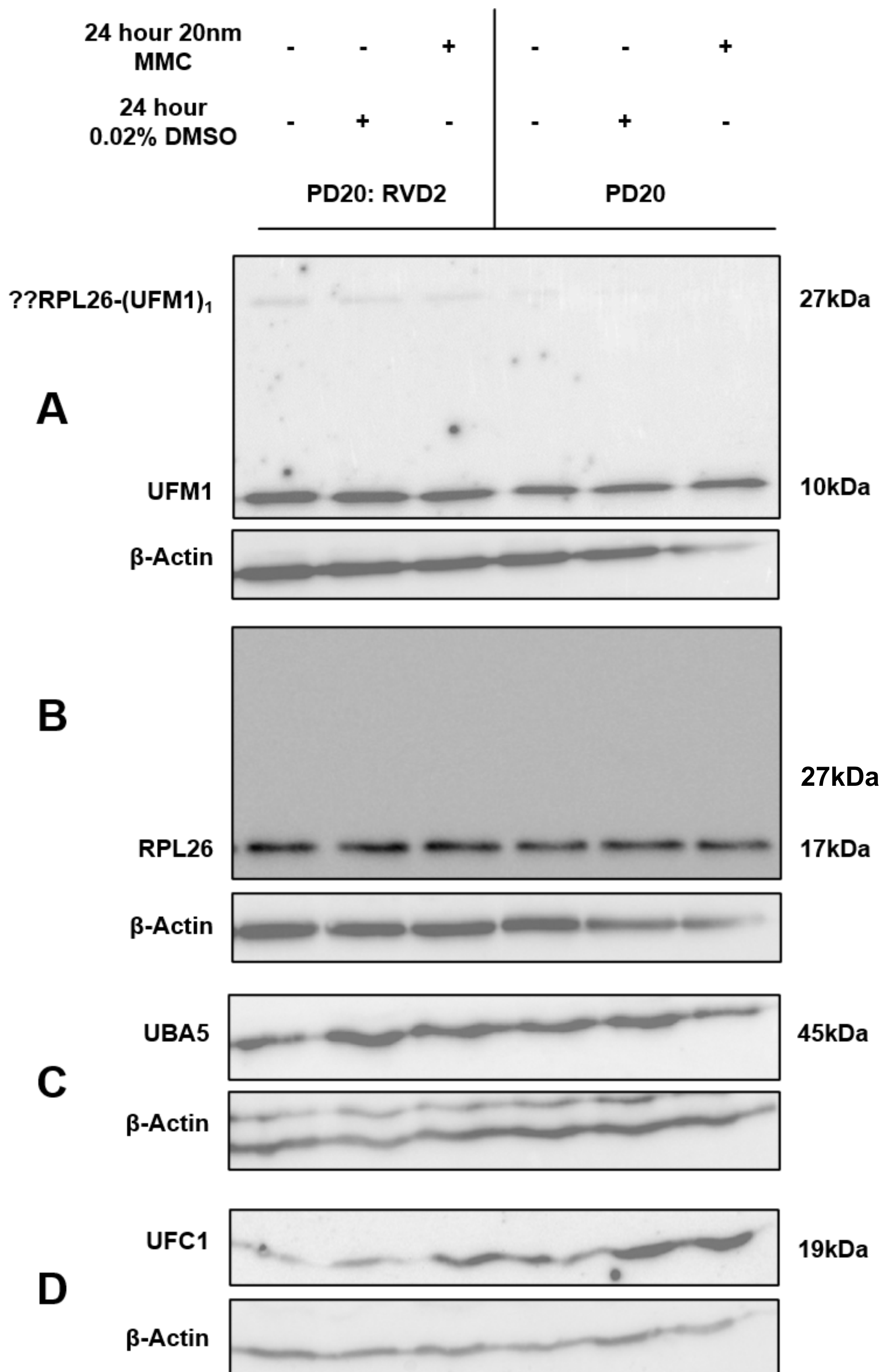

*Figure 13 – **MMC/DMSO Treated Fibroblast UFMylation pathway activity.** 12.5% Western Blot prepared with 30µg two lymphocyte cell lines: SV40-transformed FA fibroblast cell lines: PD20 (FancD2 $^{-/-}$ mutant) and PD20 RV:D2 (FancD2 $^{-/-}$ plus RetroViral FANCD2 transgene in the presence or absence of the drugs detailed. β-Actin used as loading control. Probed with antibodies against **A** UFM1. **B** RPL26. **C** UBA5. **D** UFC1.*

The UFMylation cascade protein UBA5 showed a consistent expression between the fibroblast FA mutant and retrovirally corrected cell lines, Figure 13c. The E2 UFC1 had some variation in its expression between the PD20:RVD2 and PD20 cell lines but there

was no further antibody binding (Figure 13d). The variation observed was not able to be confirmed and further investigation would be required to confirm any differences in expression.

3.7 Preliminary Further Study

3.7.1 Cell Growth Density Impacts UFMylation Target

The varied detectable presence of UFMylation presented by the western blots probed with UFM1 and RPL26 antibodies within this study contrasts with the accepted target activity of UFMylation in literature (Walczak *et al*., 2019).

In an attempt to address these inconsistencies, any differences surrounding the cell lysate preparations and storage, as well as the western blot procedure were reviewed. Though the probing concentrations of the antibodies and western blot preparation was conducted consistently, discrepancy was found between the lymphocyte and fibroblast cell pellet preparation densities of the lysates utilised during western blot analysis (Supplementary Figure 3). This could have had an effect on cell cycle stage and the levels of replication stress within the cell populations. Though all the cell lines used (MRN; CB15-0387; PD20; PD20 RV:D2) had been prepared and theoretically maintained in logarithmic growth, there was a variety of growth stages in the suspension (lymphocyte) and adherent (fibroblast) cell lines. Cell cultures should be maintained in a logarithmic growth state to maintain the continual presence of space and nutrients for all growing cells – if cells become confluent then limited space to grow could result in cell stress and promotion of mutagenic responses (Fulda *et al.,* 2010). Therefore, the variety of growth stage of the cell lines when cells were harvested for western blot analysis could have influenced cell stress response proteins such as RPL26.

This cell stress/density theory was explored using the adherent fibroblast cell lines. The cell culture growth flasks were seeded at mid log-phase, transition phase and stationary phase concentrations: $4x10^4$ cells/ml; $8x10^4$ cells/ml and $1x10^5$ cells/ml respectively and incubated for 72-hours before harvest. Seeding concentrations were extracted from the cell growth curve produced preceding the fibroblast 96-well plate cell growth assay (Supplementary Figure 2).

At harvest, the cell flasks were observed to be 90% confluent, 100% confluent and an overgrown, respectively. These cells were processed and run on a western blot on the

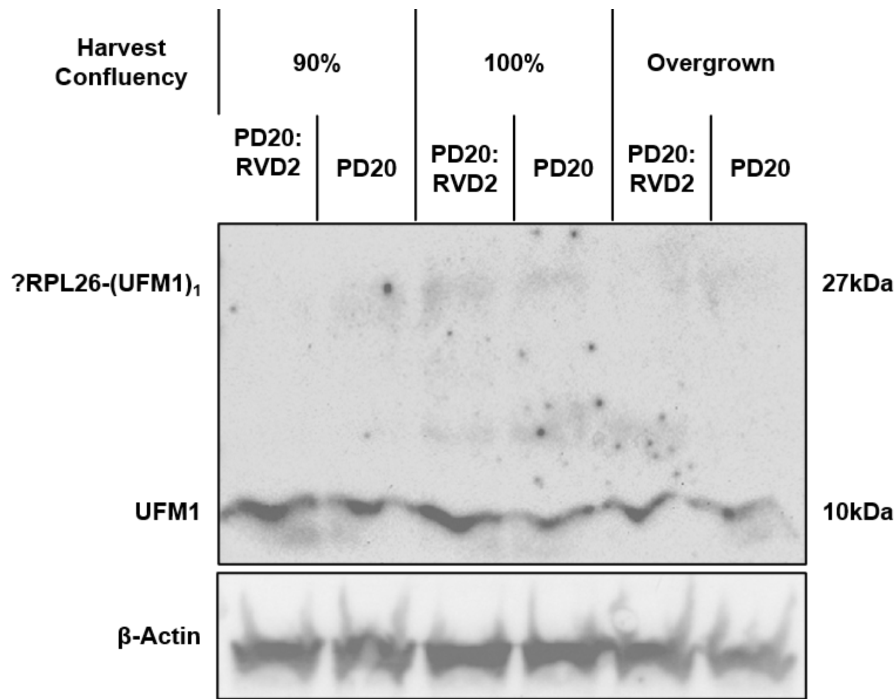

*Figure 14 – **Cell Pellet Density UFM1 Test.** 12.5% Western Blot with 30µg SV40-transformed FA fibroblast cell lines: PD20 (FancD2 $^{-/-}$mutant) and PD20 RV:D2 (FancD2 $^{-/-}$ plus RetroViral FANCD2 transgene) Lysates prepared at : 4x10^{4} cells/ml; 8x10^{4} cells/ml and 1x10^{5} cells/ml respectively then harvested at the described confluency (90%; 100%; Overgrown respectively) following 72 hour incubation to test cell density impact on protein presence. Probed with UFM1 antibody, β-Actin used as loading control.*

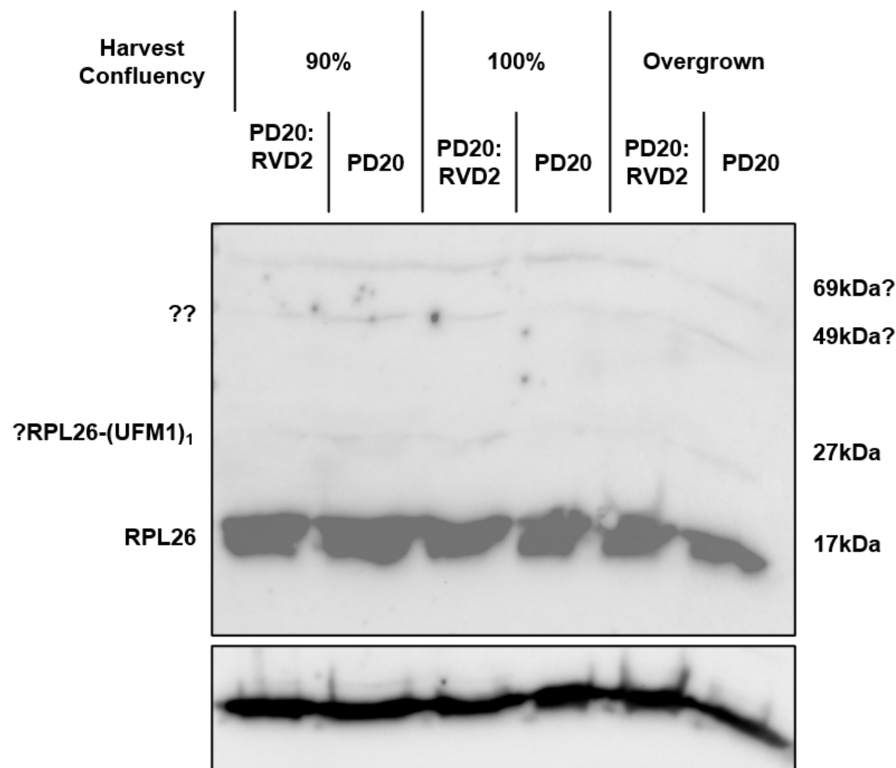

*Figure 15 – **Cell Pellet Density RPL26 Test.** 12.5% Western Blot with 30µg SV40-transformed FA fibroblast cell lines: PD20 (FancD2 $^{-/-}$mutant) and PD20 RV:D2 (FancD2 $^{-/-}$ plus RetroViral FANCD2 transgene) Lysates prepared at : 4x10^{4} cells/ml; 8x10^{4} cells/ml and 1x10^{5} cells/ml respectively then harvested at the described confluency (90%; 100%; Overgrown respectively) following 72 hour incubation to test cell density impact on protein presence. Probed with RPL26 antibody following one freeze/thaw instance, β-Actin used as loading control.*

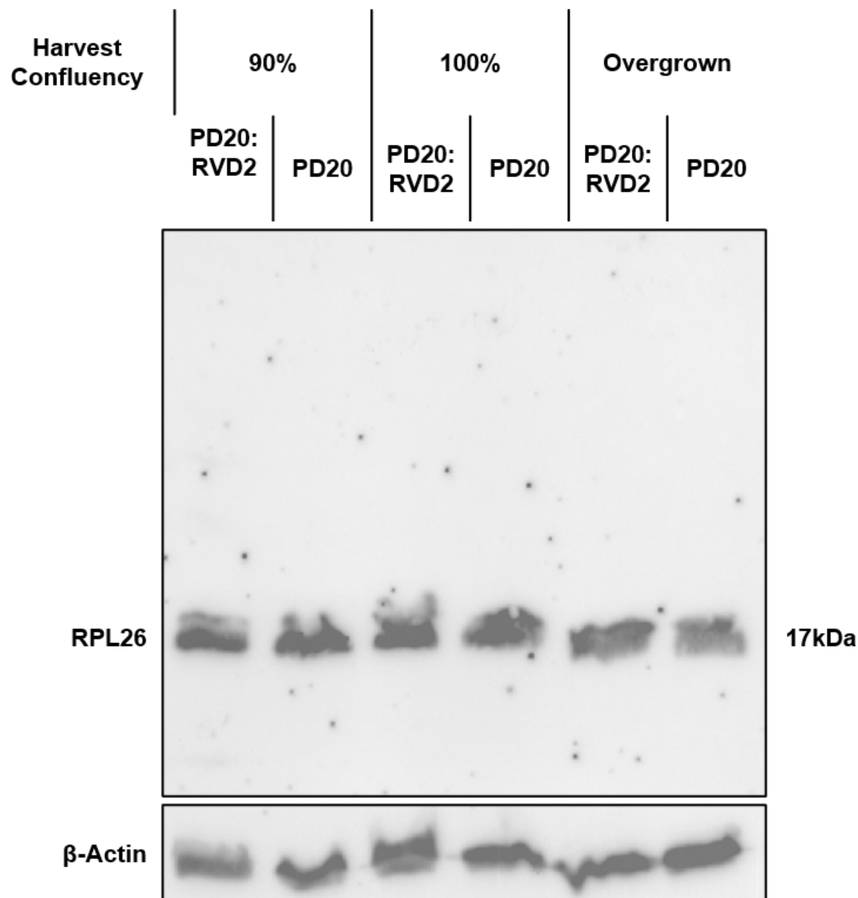

*Figure 16 – **Cell Pellet Density RPL26 Repeat.** 12.5% Western Blot with 30μg SV40-transformed FA fibroblast cell lines: PD20 (FancD2 $^{-/-}$mutant) and PD20 RV:D2 (FancD2 $^{-/-}$ plus RetroViral FANCD2 transgene) Lysates prepared at : 4x10^{4} cells/ml; 8x10^{4} cells/ml and 1x10^{5} cells/ml respectively then harvested at the described confluency (90%; 100%; Overgrown respectively) following 72 hour incubation to test cell density impact on protein presence. Probed with RPL26 antibody following two freeze/thaw instance, β-Actin used as loading control.*

same day without freezing, Figure 14. Interestingly, a secondary UFM1 band at 27kDa is only present in the 100% confluent lysate (set up at 8x10^{4} cell/ml, harvested at 100% confluency) which following 72-hours incubation within the cell growth analysis (Supplementary Figure 3) suggests the cell are in a transitionary growth phase from logarithmic to lag (Jaishankar & Srivastava, 2017). This stage could introduce cellular stress factors due to reduced nutrient availability (Gameiro & Struhl, 2018). Time did not allow for explicit analysis of cell cycle status or measurement of oxidative stress markers (Filomeni, De Zio & Cecconi, 2014).

A RPL26 probed western blot, produced with the same lysates following one freeze/thaw cycle, Figure 15, suggests there are secondary and tertiary RPL26 presence in all cell density lysates that had not previously been observed in this study with fibroblast cell lines. The lysates were freeze-thawed to investigate the effect of $-20℃$ storage on protein integrity (Wöll & Hubbuch, 2020). A western blot prepared

with the cell density lysates following two freeze/thaw cycles post lysis, Figure 16, did not demonstrate the additional antibody binding bands that previously demonstrated in Figure 15. This suggests that the PTM of RPL26 breaks down following two freeze-thaw cycles however, this was only tested in one repeat and so further investigation is required.

3.7.2 Lower NaCl Concentration for More Efficient RPL26 Immunoprecipitation

Given the reported association of UFM1 and RPL26 in the literature, immunoprecipitation (IP) studies were started to analyse their interaction in the FA and non-FA cell lines.

Figure 17 demonstrates an IgG isolate heavy and light chain presence following an RPL26 isolate prepared with an 200nM NaCl IP buffer. The high presence of unbound RPL26 in both the FA and the retrovirally corrected cell lines following western blot probing with RPL26, suggested that there was limited protein isolated. An alternative IP lysate with a lower NaCl concentration (50mM), to reduce the destabilisation of protein interactions during lysing (Logisz & Hovis., 2005), was prepared and western blot analysis conducted with the new lysates, Figure 18. The lower NaCl concentration lysis buffer yield a more successful RPL26 isolate though some unbound RPL26 is still present. Due to time restraints the reduced NaCl lysate RPL26 IP samples were not probed with a UFM1 antibody. This would have demonstrated whether, within these lysates, RPL26 is modified by UFM1 as the literature suggests.

Preliminary IP investigation began into the potential interaction of UFM1 with poor AML prognostic factor EVI1. EVI1 has been shown to be upregulated in individuals with FA increasing their risk of poor prognostic AML (Meyer *et al*., 2007). The EVI1 IP lysates prepared in 200mM IP buffer did suggest the successful isolation of EVI1, Figure 19. Interestingly, EVI1 was not observed in the input lysate despite adjusting loading volumes to maintain equivalent concentrations of protein, though this could be associated with cell line immortalisation techniques. Due to time constraints, probing with UFM1 antibodies was not able to be conducted with these lysates.

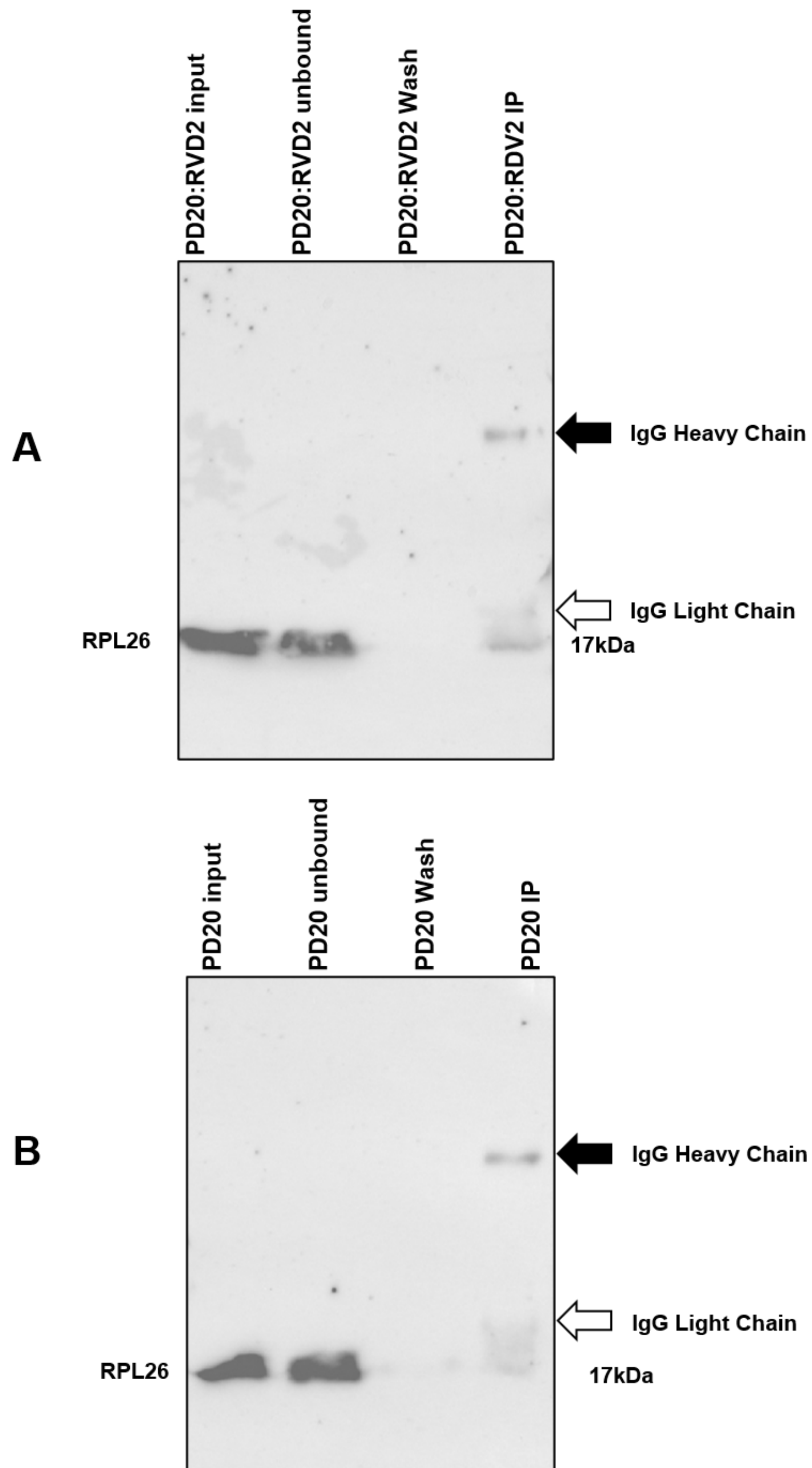

*Figure 17 – **RPL26 IP with 200mM NaCl IP buffer.** 12.5% RPL26 Immunoprecipitation (IP) Western Blot prepared with SV40-transformed FA fibroblast cell lines prepared in 200nM NaCl IP lysis buffer. Loaded with 30μg or equivalent of input lysates, unbound supernatant, wash and RPL26 isolate **A** PD20 RV:D2 (FancD2$^{-/-}$ plus RetroViral FANCD2 transgene) and **B** PD20 (FancD2 $^{-/-}$ mutant).*

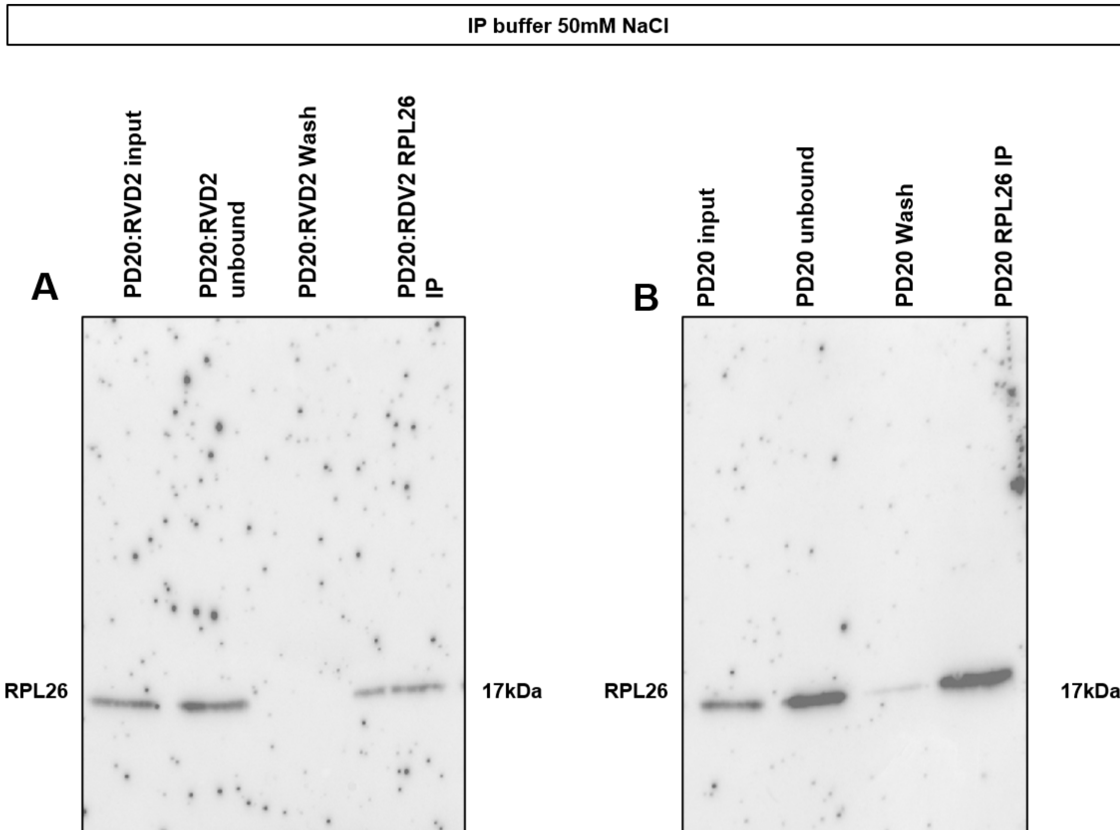

*Figure 18 - **RPL26 IP with 50mM NaCl IP buffer** 12.5% RPL26 Immunoprecipitation (IP) Western Blot prepared with SV40-transformed FA fibroblast cell lines prepared in 50nM NaCl IP lysis buffer. Loaded with 30µg or equivalent of input lysates, unbound supernatant, wash and RPL26 isolate **A** PD20 RV:D2 (FancD2 $^{-/-}$ plus RetroViral FANCD2 transgene) and **B** PD20 (FancD2 $^{-/-}$mutant).*

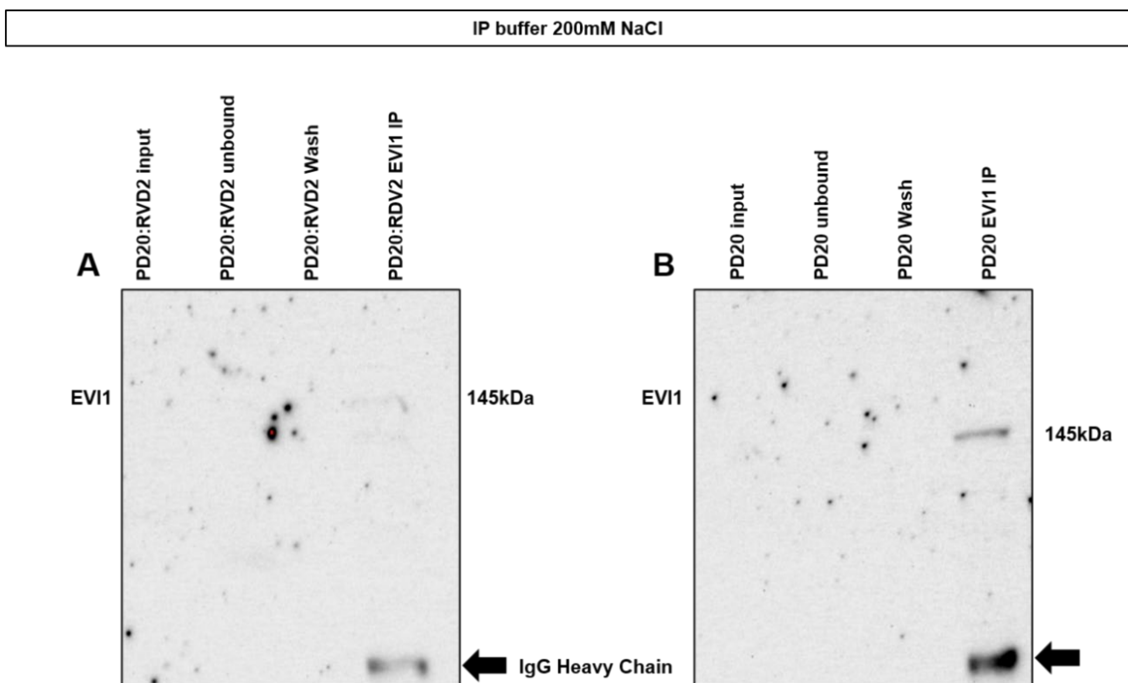

*Figure 19 – **EVI1 IP with 200mM NaCl IP buffer** 7.5% EVI1 Immunoprecipitation (IP) Western Blot prepared with SV40-transformed FA fibroblast cell lines prepared in 200nM NaCl IP lysis buffer. Loaded with 30µg or equivalent of input lysates, unbound supernatant, wash and EVI1 isolate **A** PD20 RV:D2 (FancD2 $^{-/-}$ plus RetroViral FANCD2 transgene) and **B** PD20 (FancD2 $^{-/-}$mutant).*

4. Discussion

Preliminary western blot data, by Pierce *et al*. (Unpublished) prepared several lymphocyte FA mutant and non-FA cell lines to explore UFM1 presence and any potential UFMylation activity. They observed UFMylation activity restricted to the FA mutant cell line in line at 27kDa, which was consistent with UFMylated RPL26. This project initially aimed to reproduce Pierce *et al*'s data and extend the understanding around the implication of UFMylation activity related to DNA damaging agents such as MMC or radiation.

The accepted literature suggests that RPL26 is the primary target of UFMylation and responds to cellular and ER stress to implement ER-phagy (Walczak *et al*., 2019). UFMylation activity is highly conserved in eukaryotic cells and has been presented in Human Embryonic Kidney (HEK) 293T cell line; human osteosarcoma U2OS; epithelial-like human breast cancer MDA-MB-231 cell line; pancreatic (PANC or PaCa) cancer cell lines PANC-1 and Mia PaCa-2 (Jiang *et al*., 2023; Zhao *et al*., 2020; Qin *et al*., 2019). UFMylation has also been demonstrated to be essential for the differentiation of lymphoid resident antibody-secreting B cells and the survival of haematopoietic stem cells (Millrine *et al*., 2023; Lee *et al*., 2021). Pierce *et al*., (Unpublished) suggested that RPL26 UFMylation activity takes place at a higher level in FA-mutant cells, contrasting considerably with the accepted literature. This study was able to demonstrate UFMylation activity believed to be UFMylated RPL26 across both FA-mutant and non-FA cell lines.

The behaviour of the FA mutant and non-FA mutant cell lines witnessed within this study was consistent with accepted UFMylation activity however, the contrast of the lymphocyte cell lines with the activity of the preliminary data of Pierce *et al*., lead to the investigation of their FANCD2 status. Western blot analysis with aFANCD2 antibody prompted the suggestion that a mutation had potentially taken place in the lymphocyte “non-FA-mutant” cell line MRN. A presence of FANCD2 in the MRN cell line was not detected nor was a significant differential sensitivity to the DNA damaging agent MMC demonstrated. Resistance to MMC would be expected of a non-FA cell line as it retains the capacity to compile the FA pathway and repair ICL induced ds-DNA damage (Hammarsten et al., 2021). There was no alternative lymphocyte non-FA mutant cell line available at the time of the study so investigations were altered for

a SV40-fibroblast cell line. Nonetheless, the data produced by the lymphocyte FA mutant, CB15-0387, should not be discarded. The *FancP/SLX4* mutant demonstrated an appropriate MMC sensitivity as expected of an FA cell line. Kim *et al*. (2013) demonstrates within their 8-day MMC incubation assay, a SLX4-ΔSAP cell line that exhibits a similar 33% survival as CB15-03087 (*FancP/SLX4* mutant) when treated with 50nM MMC. The CB15-0387 cell line replicated the UFMylation activity suggested by Pierce et *al*., (Unpublished) to be UFMylated RPL26, with additional suggestion that treatment with DMSO and MMC impacts the UFMylation activity to contrasting effects.

In some instances, an increased expression of UFMylated protein following DMSO exposure was observed in the FA lymphocyte cell line probed with UFM1 antibody. This add to the speculation that UFMylation of RPL26 is a cell stress response as DMSO is a strong aprotic solvent that enhances solubility of the transmembrane (Papich, 2016). DMSO is known to induce oxidative stress within cellular experimentation models (Dludla *et al*., 2021). Increased UFMylation following DMSO treatment could have suggested a mechanism where UFM1 preferentially binds to a protein and therefore increases UFMylation activity during times of cellular stress. However, the DMSO effects were not replicated in the fibroblast cell lines tested when they were probed with UFM1 antibody or other UFMylation pathway components. Interestingly, the detectable levels of UFMylation activity when probed with UFM1 antibody did not increase following additional exposure to MMC dissolved in DMSO compared to the FA cell line's respective DMSO treatment. This may reflect the already stressed cellular environment within FA cell line due to its increased susceptibility to ICL DNA damage, especially following MMC exposure (Francies *et al*., 2018).

Noticeably the UFMylation activity was only demonstrated when western blots were probed with an UFM1 antibody (and not the RPL26 antibody) in the CB15-3087 (*FancP/SLX4* mutant) and fibroblast cell lines (RVD2:PD20 - retrovirally corrected *FancD2* mutant and PD20 - *FancD2* mutant). Antibody binding that would highlight PTM activity of RPL26 was not readily observed when western blots were probed with an RPL26 antibody. However, UFM1 probed western blot observations of RPL26-UFMylation have been confirmed by other researchers with mass spectrometry analysis (Wang *et al*., 2020). Wang *et al*. (2020) suggests that RPL26 can be UFMylated at two lysine residues leading to several antibody bands when a western

blot is probed with UFM1 antibody. One could speculate that the RPL26 antibody utilised within this study has a binding site that is limited following UFMylation activity or that the RPL26 protein changes its tertiary structure following UFMylation, obscuring the RPL26 antibody binding site.

There is some evidence to suggest that trypsinisation of adherent cells from a growth flask leads to the removal of membrane proteins. Huang *et al*. (2010) suggests that both cell surface and mitochondrial surface proteins are destroyed by trypsinisation whilst proteins associated with cell apoptosis are up regulated. Within this study the harvested fibroblast cells were washed several times in PBS following trypsinisation and a neutralisation with FBS. The cell pellets were lysed in RIPA buffer or IP buffer immediately following washing which differed from the 2-hour rest period allowed for both lymphocyte and fibroblast cells following seeding in a 96-well plate before MMC drug treatment was applied. Literature suggests that cellular morphology and protein activity recovers 24-48 hours after trypsin exposure (Sharma *et al*., 2019; Huang *et al*., 2010). It would be interesting to explore the impact of a rest and recovery period preceding lysing on UFMylation activity in fibroblast cells following trypsinisation.

Alternatively, the antibody binding ability could be affected by freeze-thaw maintenance of cell lysates or the antibody itself as it is known to lead to protein degradation (Wöll & Hubbuch, 2020). This is widely understood in the storage and use of protein lysate samples but the implied limitations within PTM of protein lysates had not previously been explored by Pierce *et al*. (Unpublished) in their investigation of FA UFMylation activity. However, there is evidence to suggest that thioester bonds, such that UFM1 forms with its targets, are not denatured in reducing conditions during lysate preparation for western blot analysis and can be visualised (Kumar *et* al, 2021; Holm *et al*., 2012). PTM of RPL26 with UFM1 is associated with a covalent bonding at lysine residues Lys132 and Lys134 which also does not disassociate during the boiling process (Scavone *et al*., 2023; Walczak *et al*., 2019).

The consideration of freeze-thaw maintenance and the differentiation within preceding western blots lead to cell density testing being conducted in this study. Cell density testing was utilised to explore the impact of cellular stress on the induction of UFMylation activity. RPL26 is theorised to be a cell stress response protein promoting autophagy of the ER following cellular stress and therefore the battle for nutrients

within a confluent cell flask could encourage autophagy process (Scavone *et al*., 2023; Hu *et al*., 2020). There was some evidence within the cell density testing to suggest that confluency of growing cells did have an impact on UFMylation activity when probed with an UFM1 antibody. But subsequent testing with RPL26 antibody on the same lysates did not demonstrate this discrepancy. In some instances, overgrowth of cells within a growth flask can result in cell cycle stalling due to nutrient deprivation (Chen *et al*., 2012). This was not explicitly explored within this study but the use of Flow Activated Cell Sorting (FACS) could extend the cellular density investigation. FACS would allow researchers to quantify the relative stages of cell cycle in flasks of different cell densities (and therefore different observed levels of UFMylated RPL26) to investigate whether UFMylation of RPL26 is a cell stage dependent mechanism (Yiangou *et al*., 2019; Liao *et al*., 2016). Review of the UFMylation target literature and the understood mechanisms of the UFMylation pathway cumulates to suggest that UFM1 acts as a signal transducer for reactions like a PTM with a phosphate group. This differs from the role of mono- or poly- addition of UFM1's namesake ubiquitin, which highlights a protein for amplification or degradation (Komastu *et al*., 2004).

Continuation of the IP analysis begun within this study would need to consider the results of aforementioned tests to accurately reflect the UFMylation activity occurring. An alternative RPL26 antibody in further investigations might strengthen western blot analysis and IP isolation. IP methods began to isolate the AML prognostic marker EVI1. EVI1 is a zinc finger transcription factor associated with high-risk poor prognosis in both FA and non-FA derived AML (Paredes *et al*., 2020; Dutta *et al*., 2013). EVI1 is not a confirmed target of UFMylation but IP analysis could highlight an interaction.

5. Conclusion

This project successfully demonstrated UFMylation and associated protein activity with the overall elements disagreeing with Pierce *et al*'s unpublished data which suggested that UFMylation of RPL26 takes place only in FA cell lines. This study conducted an analysis of the UFMylation literature which cumulated to suggest that the PTM is a signal transducer involved in many intracellular pathways and is reportedly preserved across eukaryotic cell lines. This analysis contributed to wider considerations within the western blot interpretation of this study.

This thesis endeavoured to investigate the effect of induced cellular stress had on UFMylation activity. We aimed to study the effect of several cell stress inducers including irradiation on activity but we only utilised the ICL inducing drug MMC. We treated cell cultures for drug dose sensitivity testing and cell growth flasks for western blot analysis with MMC or its carrier DMSO, though neither treatment demonstrated a significant impact on UFMylation activity within this study. However, this posed the opportunity to suggest that UFMylation does not interact or impact the FA pathway. Drawing from the suggestion that UFMylation is a signal transducer with additional understanding of the isolated reaction pathways in each mitotic stage, the project progressed to consider the growth conditions of the cells utilised and the reflective cell stress caused by limited growth resources in confluent conditions that could contribute to the UFMylation of RPL26. The data did demonstrate that confluency of the cell growth flask impacts the detected level of UFMylation activity with RPL26. Initial study was also commenced to identify whether EVI1 is a target of UFMylation but further refinement of the isolation lysis would be required for continued investigation.

This study contributed to the understanding of UFMylation within a FA mechanism that had not previously been conducted. We began to demonstrate that UFMylation does not act independently within an FA system but further study with alternative FA mutant cell lines would strengthen the understanding of UFMylation interaction in different areas of the FA pathway. The importance of precision within cell culture management and lysate preparation was highlighted within the UFMylation activity investigation as different growth phrases provide a different combination of PTM that can lead to unique results.

6. Future Work

Continued investigation into the role of UFM1 addition to a protein is required. Here, UFMylation is theorised to have a role as a signal transducer but this is yet to be investigated explicitly. Analysis of the influence of cellular stage, intracellular stress, and induced stress, including irradiation, on UFMylation could aid in the answer of these requirements. RPL26 UFMylation would benefit from a detailed study of differential levels throughout the cell cycle. Additionally, bioinformatic rendering of RPL26 tertiary structure rearrangement following PTM could refine understanding of its own role and that of UFMylation.

7. Methodology

7.1 Cell Culture

Lymphocyte cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 20% foetal bovine serum (FBS) (Hyclone) and 4mM L-Glutamine (Gluta-MAX) (Thermo Fisher). The cells were sub-cultured when at $1x10^6$ cells/ml in suspension down to $2x10^5$ cells/ml. All cell groups were incubated at 37.5°C in humidified 5% CO_2.

PD20 ($FANCD2^{-/-}$); PD20 RV:D2 (FANCD2-/- plus retrovirally corrected FANCD2 transgene) fibroblast cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% FBS (Hyclone or Gibco). Once 80-95% confluent the adhered cells were washed in Dulbecco's Phosphate Buffered Saline (PBS) (Sigma-Aldrich) then a minimum sufficient volume of 0.25% Trypsin – Ethylenediaminetetraacetic acid (EDTA) (Gibco) was used to dissociate the adherent cells from the tissue culture flask and from each other. An equal volume, to the Trypsin-EDTA, of FBS was added to deactivate the protease activity of trypsin before splitting to 15-20% confluency to maintain logarithmic growth. Both cell lines were incubated at 37.5°C in humidified 5% CO_2.

7.2 Drug Treatment

Cell preparations treated with 20nM (100μM stock) mitomycin C (MMC) or equivalent dilution of Dimethylsulfoxide (DMSO) (100% stock) (PanReac AppliChem) and incubated at 37.5°C in humidified 5% CO_2 for time indicated prior to collection. Fibroblast cell lines were subject to an additional step of trypsinisation and FBS neutralisation for collection. The pellets were centrifuged at 350g for 5 minutes and washed 3 times in PBS, during final wash the cell pellet was transferred to an Eppendorf. Following a final centrifugation step the PBS is removed and the pellet dried before storing at -20°C if required.

7.3 Cell Lysis

7.3.1 RIPA

Mid log phase cells were washed twice in PBS lysed in RIPA lysis buffer (RIPA buffer (50mM Tris-HCl; 150mM NaCl; 1% Sodium deoxycholate; 1% NP40; 0.1% SDS; 1mM EDTA); plus 10μL/mL Phosphatase inhibitors; 10μL/mL protease inhibitors; 10μL/mL Sodium Orthovanadate; 1.5μL Benzonase). Lymphocyte cells were lysed in 50μL RIPA

buffer per $1x10^6$ cells. The fibroblast cell lines were treated the same and lysed in 25µL RIPA buffer per $1x10^6$ cells.

Cells were incubated on ice for 20 minutes followed by centrifugation at 24000g at 4°C for 10 minutes to pellet cell debris. The supernatant was harvested as cell lysates.

7.3.2 Immunoprecipitation

Mid log phase cells were washed twice in cold PBS and lysed in IP buffer (200mM NaCl; 50mM Tris; 1mM EDTA; 1% Triton-X100; 2mM $MgCl_2$) with 10µL/mL Phosphatase inhibitors; 10µL/mL protease inhibitors; 1.5µL Benzonase. Fibroblast cell lines were lysed with 500µL lysis buffer per $1x10^7$ cells. An alternative IP buffer was prepared for RPL26 lysate isolate with 50mM NaCl.

Cells were incubated 4°C for 30 minutes with rotation, then sonicated 3x5 seconds in a water bath with another 4°C incubation for 30 minutes. The lysed cells were then centrifugated at 24000g for 10 minutes at 4°C to pellet cell debris. The supernatant was harvested as cell lysates.

7.4 Protein Assay

Serial dilution of 0.25mg/ml Bovine Serum Albumin (BSA) prepared as standards with 0; 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5µg in 80µl per well. Each standard was plated in triplicate on a 96 well plate. Lysate samples were individually prepared at 1/10 and 1/20 dilutions and plated in triplicate on the 96 well plate. 20µl BioRad Protein Dye was added and absorbance measured immediately at 570nM. Protein concentrations were read with reference to the standard curve, lysate dilutions were prepared to ensure sample concentrations were on the standard curve.

7.5 Cell Dilution Proliferation Assay

Appropriate cell concentration and WST-1 incubation time was established using a multi-day cell dilution proliferation assay. Flat bottom, non-pyrogenic polystyrene treated, sterile tissue culture 96-well plates (CoStar Corning Incorporated) were prepared with 100µL of the appropriate cell dilutions: $5x10^3$ cells/ml; $7.5x10^3$ cells/ml; $1x10^4$ cells/ml; $2.5x10^4$ cells/ml; $5x10^4$ cells/ml; $7.5x10^4$ cells/ml; $1x10^5$ cells/ml. Separate plates were prepared for 24; 48; 72 hours and cumulative absorbance measured at a wavelength of 450nM was taken hourly, over 4 hours, following 10µL/well administration of WST-1 reagent on the appropriate day.

7.6 MMC Drug Dose Proliferation Assay – WST-1

The 96-well plates were seeded with 100µL of $5x10^4$ cell/ml and $4x10^4$ cells/ml suitable to the cell lines and left to settle for up to two hours before treatment with 1µL of 0nM-150nM concentration of Mitomycin C (MMC), prepared in a dilution series with DMSO (PanReac AppliChem) – ensuring the DMSO concentration did not exceed 1%. The treated 96-well plates were incubated for 72-hours at 37.5°C in humidified 5% CO_2, later lymphocyte assays were incubated for 96-hours. The metabolic activity of the cells was assessed at 450nM following 3-hour incubation with WST-1 reagent. This assay was utilised to quantify proliferation and assess the sensitivity of cell lines to the MMC drug (Kang, Yoon & Choi., 2017).

7.7 Western Blot

Western blot techniques were used to analyse treated and non-treated cell lysates (lysed with RIPA buffer). 30µg of cell lysate were separated on 12.5%, 7.5% or 6% polyacrylamide separating gels, depending on investigative molecular weight, with a 5.5% stacking gel in a BioRad Mini PROTEAN Tetra System. Gels were run at 100V until the dye front was at the bottom of the gel in a 10% sodium dodecyl sulphate (SDS) running buffer. A Thermo Scientific™ Spectra™ Multicolor Broad Range Protein Ladder was used for comparative estimation of molecular weight of proteins and to monitor the progress of electrophoresis through the gel. The separated proteins were initially transferred onto nitrocellulose membrane in a 45-minute transfer in transfer buffer (10% methanol and 1x running buffer without SDS) at a constant 100V. When studying proteins below 100kDa transfer was achieved with transfer buffer with 1x Tris-Glycine running buffer without SDS and 20% methanol run for 60 minutes. When studying proteins above 100kDa, proteins were run on 6% separating gels with 5.5% stacking gel and transferred for 60 minutes in 1x transfer buffer without methanol.

The nitrocellulose membranes were blocked in 3% milk powder dissolved in 1x Tris buffered saline (TBS) with 0.3% Polysorbate 20 (Tween/T) for at least 1 hour then the primary antibody, see Table 1 for details, was exposed in 1% milk powder dissolved in 0.3% TBS-T overnight. The following day the excess primary antibody was washed off the membrane in at least 3 wash cycles with 0.3% TBS-T and the secondary antibody, that had conjugated horseradish-peroxidase (HRP), see Table 1 for details, was prepared in 1% milk powder dissolved in 0.3% TBS-T and was allowed to incubate for

at least 1 hour. The excess secondary antibody was washed off the membrane in at least 3 wash cycles with 0.3% TBS-T. The membranes were then incubated in Enhanced Chemiluminescence Western Blotting Substrate (ECL) (1:1 Thermo Fisher) that reacts with the HRP bound to the secondary antibody on the membrane producing chemiluminescent reactions under UV and blue wavelengths captured by the ChemiDoc (BioRad) for up to 30 minutes.

After imaging the membranes were washed several times in TBS-T and exposed to HRP conjugated anti β-actin antibodies in 1% milk powder dissolved in 0.3% TBS-T for 1 hour. The membranes were washed again in 0.3% TBS-T and exposed to ECL and imaged for up to 3 minutes.

7.8 Immunoprecipitation

Cell lysates (IP lysis buffer unless stated) were adjusted with IP buffer to a concentration of 4mg/ml (2mg total protein in 500 μL total) (30μL of sample stored as Sample 1 - Input) and were incubated with 2μg antibody overnight at 4°C under rotation. Antibody treated lysates had 100μL of protein A/G Sepharose beads (washed twice in PBS and once in IP lysis buffer) added and incubated at 4°C under rotation. After 4 hours the lysates were centrifugated at 1500g for 5 minutes (supernatant stored as Sample 2 - Unbound) and the antibody bound beads were washed in 500μL pre-chilled IP lysis buffer 3 times and incubated at 4°C under rotation over 15 minutes (each supernatant was collected as Samples 3, 4, 5 – Wash 1, 2, 3). The final pellet was resuspended in 100μL of SDS Laemmli's loading buffer (1M Tris-HCl pH6.8; SDS; Glycerol; mercapto-ethanol; bromoethanol blue (1% in ethanol); distilled H_2O) and incubate at 95°C for 5 minutes (Stored as Sample 6 - IP).

Samples 1, 2, 3, 6 of the IP lysates were analysed via Western Blot, run with 30μg of protein or equivalent and probed with appropriate antibodies.

Table 1 – Details of antibodies and their supplier information utilised in this study.

Antibody	**Procedure**	**Company**	**Research Resource Identifiers**	**Dilution Factor**
UFM1 (Rabbit)	WB (12.5% ProtoGel)	Abcam	AB_109305 Lot: 1001123-1 Lot: GR3396232-3	1/1000
UBA5 (Rabbit)	WB (12.5% ProtoGel)	Proteintech	12093-1-AP Lot:00045984	1/1500
UFC1 (Rabbit)	WB (12.5% ProtoGel)	Abcam	AB_189252 Lot: GR3288000-1	1/1000
UFL1 (Rabbit)	WB (7.5% ProtoGel)	ThermoFisher	303-456A	1/5000
RPL26 (Rabbit)	WB (12.5% ProtoGel)	Biotechne	NB100-2131 Lot: A1	1/2500
Anti Rabbit IgG, HRP Linked Whole Ab	WB	GE Healthcare	NA9340V	1/5000
FancD2 (Mouse)	WB (6% ProtoGel)	Santa Cruise Biotechnology	Sc-20022 Lot: E1222	1/2000
Anti Mouse IgG, HRP Linked Whole Ab	WB	GE Healthcare	NA931V Lot: 9715064	1/5000
MRE11 (Mouse)	WB (7.5% ProtoGel), IP	Abcam	AB214 Lot: GR3277747-5	1/1000
Cyclin E (Mouse)	WB (12.5% Protogel)	Santa Cruz	Sc-247 Lot: K2519	1/1000
Cyclin A (Mouse)	WB (12.5% Protogel)	Santa Cruz	Sc-271682 Lot: F1319	1/1000
Cyclin B (Mouse)	WB (12.5% Protogel)	Santa Cruz	Sc-166210 Lot: B0917	1/1000
β-Actin-peroxidase (Mouse)	WB	Sigma-Aldrich	A3854 Lot: 034M4830V	1/50,000

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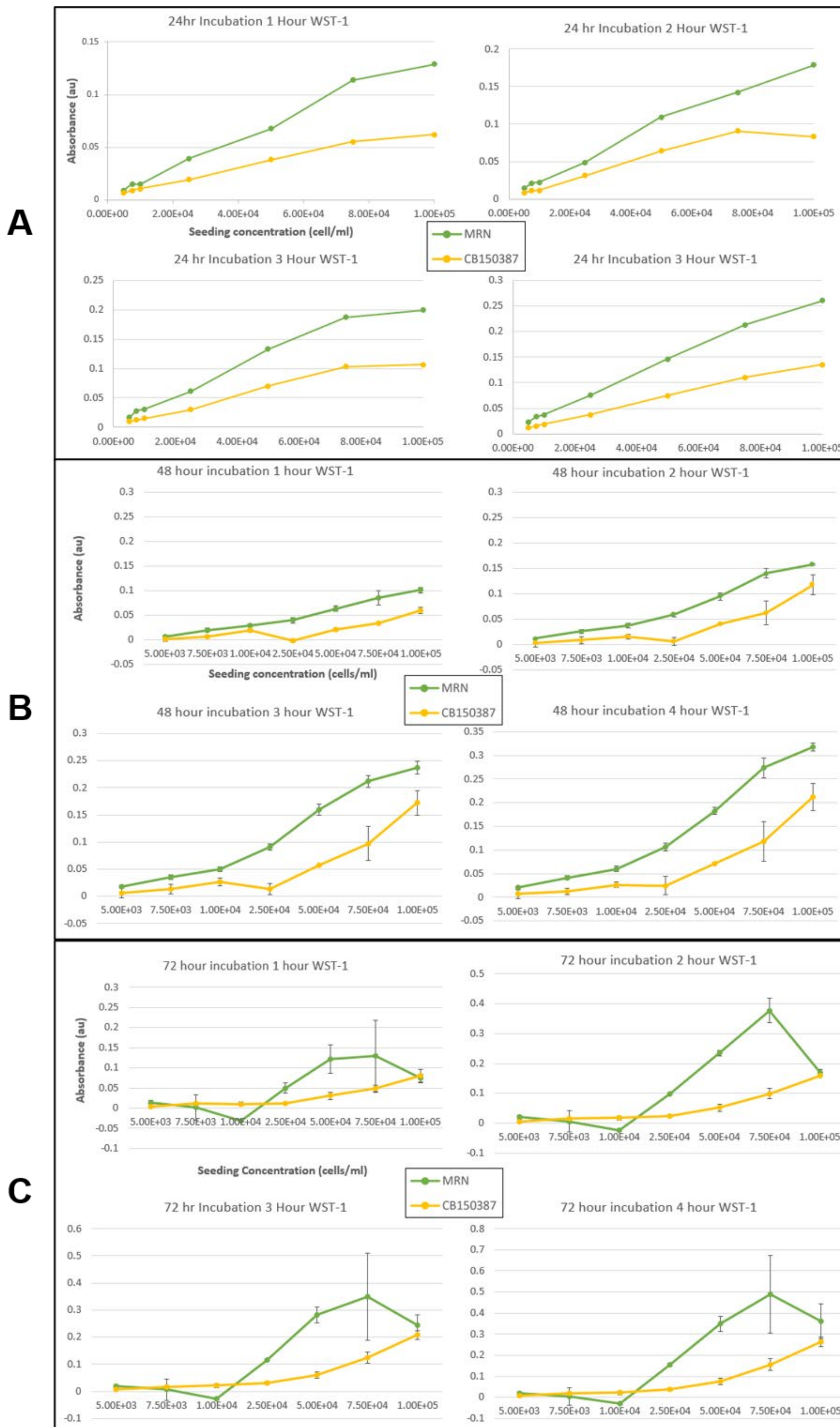
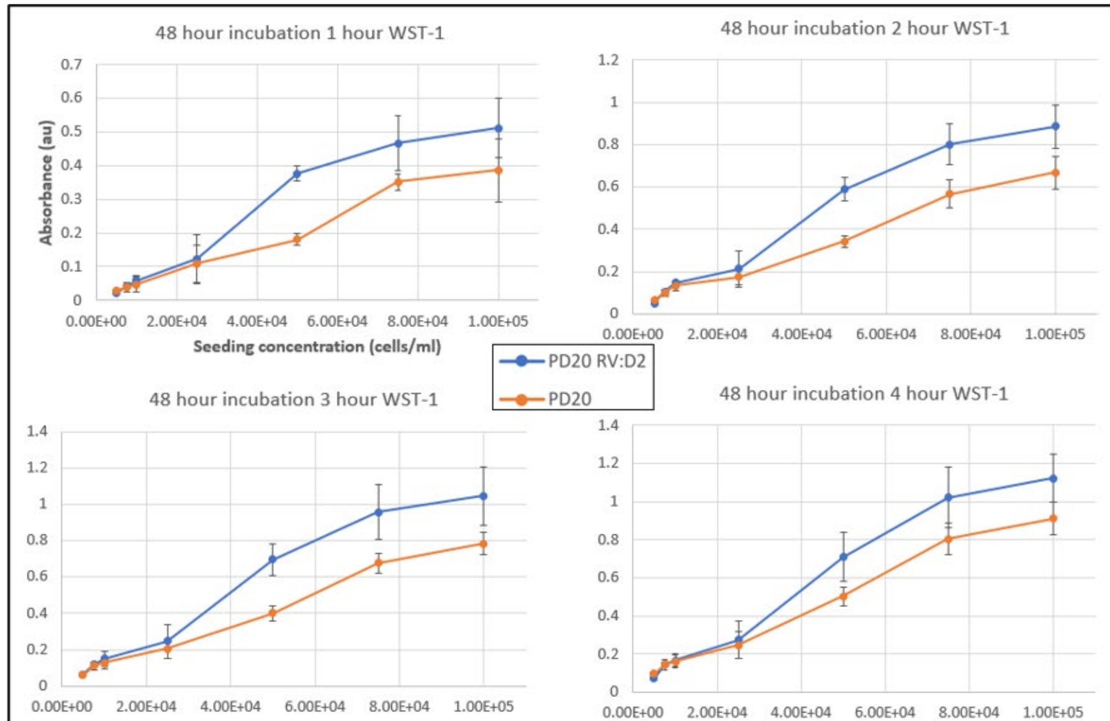
Supplementary Figures

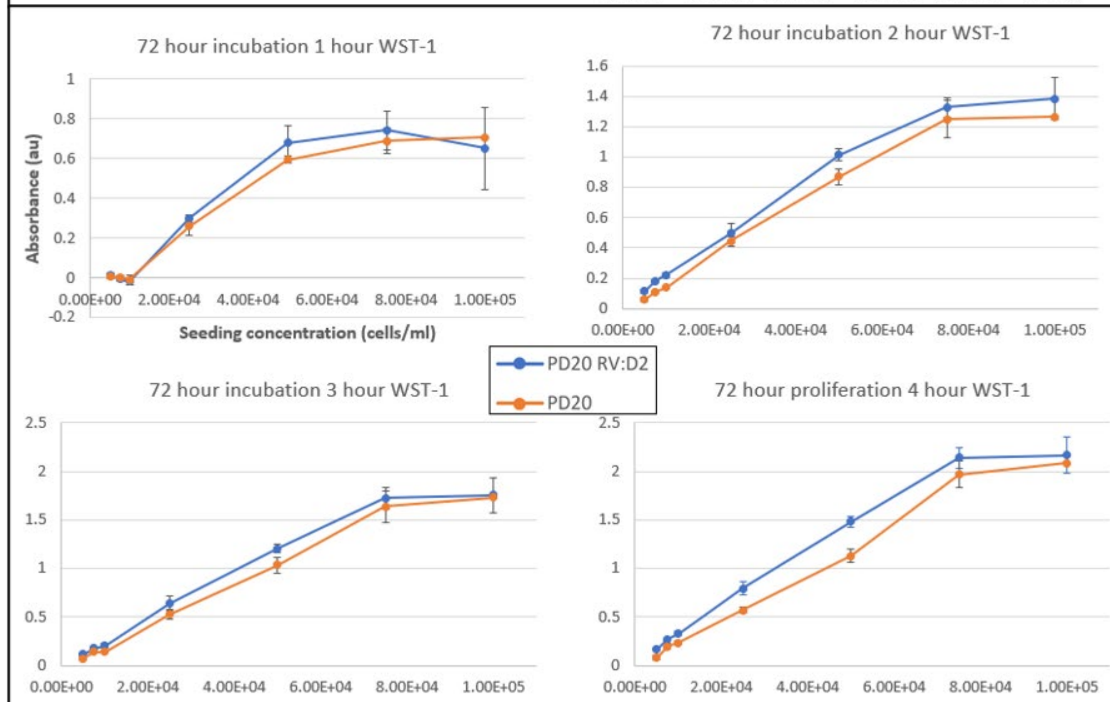


*Supplementary Figure 1 - **Multi-day proliferation assay with FA and non-FA lymphocyte cell lines.** Proliferation assay with lymphocyte MRN and CB15-0387 (FancP/SLX4 mutant) cell lines within 96-well plate to assess optimum growth concentration over 72 hours with WST-1 exposure over cumulative 3 hours.*

A

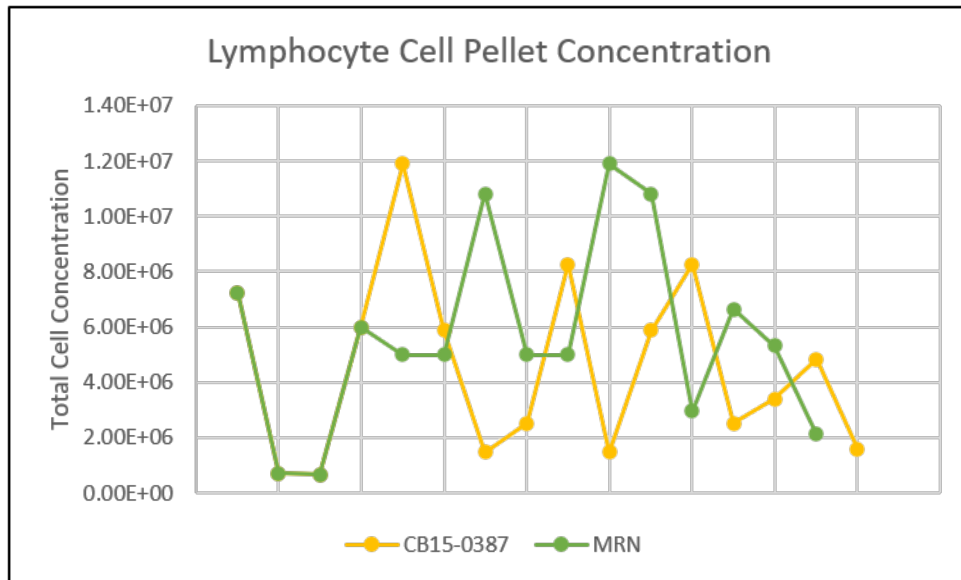


B



Supplementary Figure 2 – ***Multi-day proliferation assay with SV40-transformed FA fibroblast cell lines****. Proliferation assay with fibroblast PD20 (FancD2 $^{-/-}$ mutant) and PD20 RV:D2 (FancD2 $^{-/-}$ plus RetroViral FANCD2 transgene) cell lines within 96-well plate to assess optimum growth concentration over 72 hours with WST-1 exposure over cumulative 3 hours.*

A



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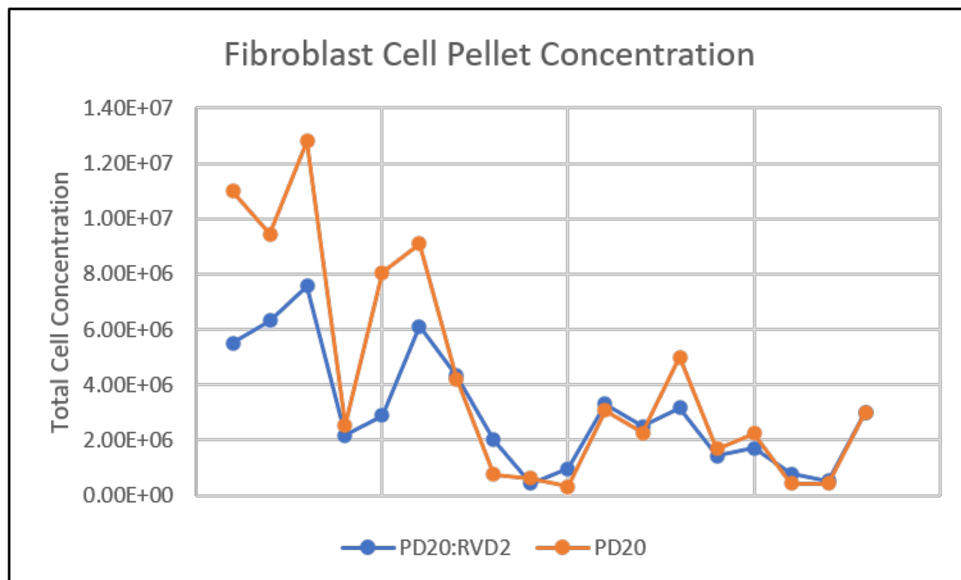


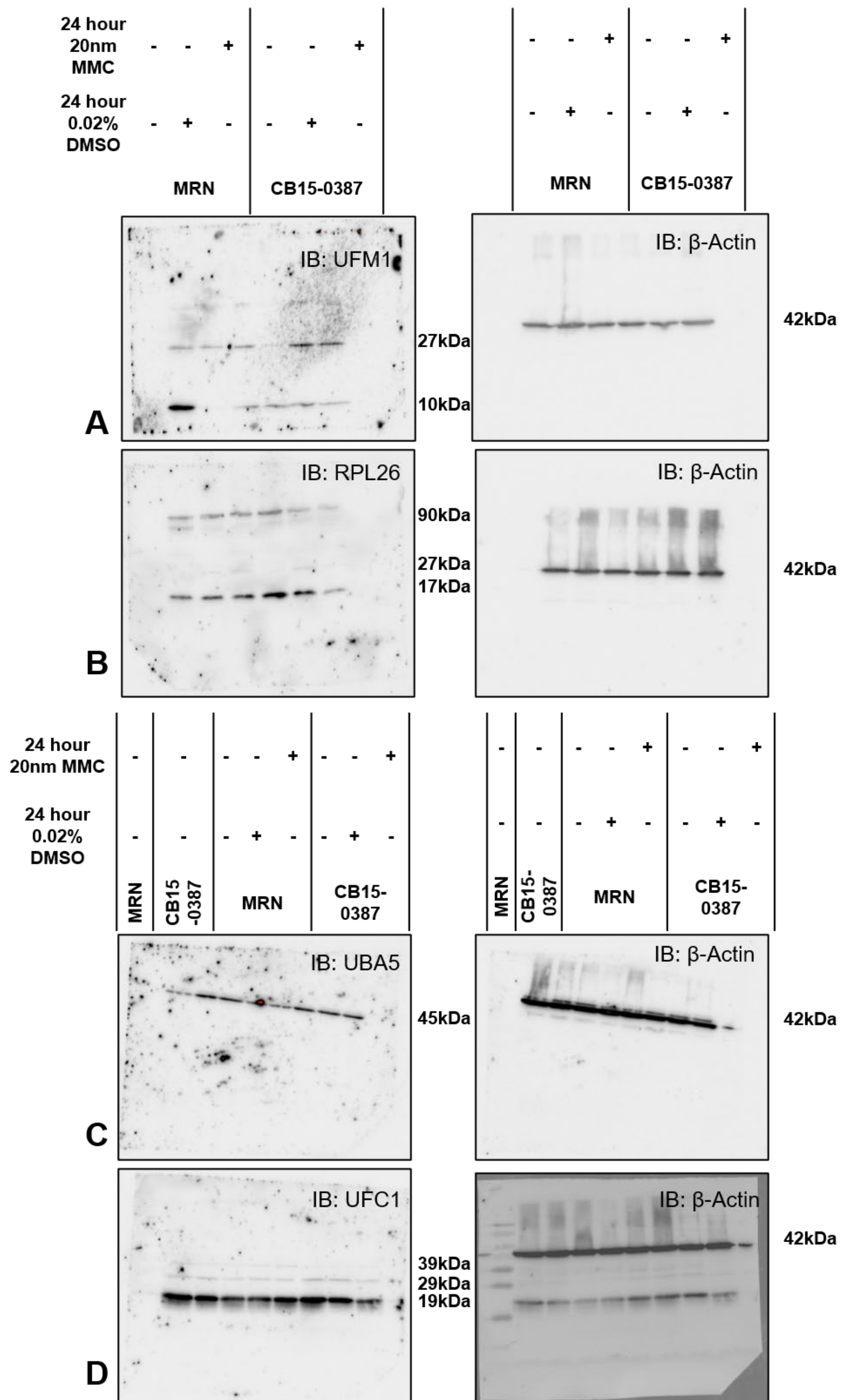
*Supplementary Figure 3 – **Cell harvest density for RIPA buffer Western Blot** lysate preparation for **A** Lymphocyte cell lines MRN and CB15-0387 (FancP/SLX4 mutant) **B** Fibroblast cell lines PD20:RVD2 and PD20 (Jakobs et al., 2012).*

Ysgoloriaethau Sgiliau Economi Gwybodaeth
Knowledge Economy Skills Scholarships

Mae'r Ysgoloriaeth Sgiliau Economi Gwybodaeth (KESS 2) yn fenter sgiliau lefel uwch Cymru gyfan a arweinir gan Brifysgol Bangor ar ran y sector AU yng Nghymru. Fe'i cyllidir yn rhannol gan raglen cydgyfeirio Cronfa Gymdeithasol Ewropeaidd (ESF) ar gyfer Gorllewin Cymru a'r Cymoedd.

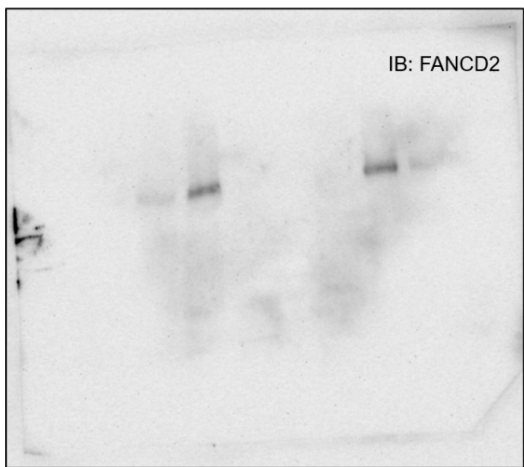
Knowledge Economy Skills Scholarships (KESS 2) is a pan-Wales higher level skills initiative led by Bangor University on behalf of the HE sector in Wales. It is part funded by the Welsh Government's European Social Fund (ESF) convergence programme for West Wales and the Valleys.

Appendix



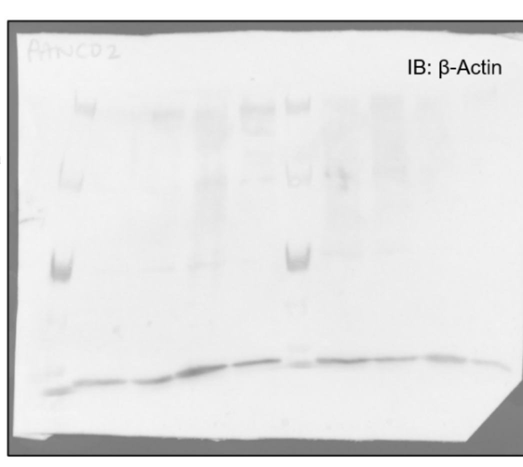
Appendix Figure 1 – Full western blot images of Figure 7.

MW	MRN	CB15-0387	PD20: RVD2	PD20	MW	MRN	PD20	CB15-0387	PD20:RVD2



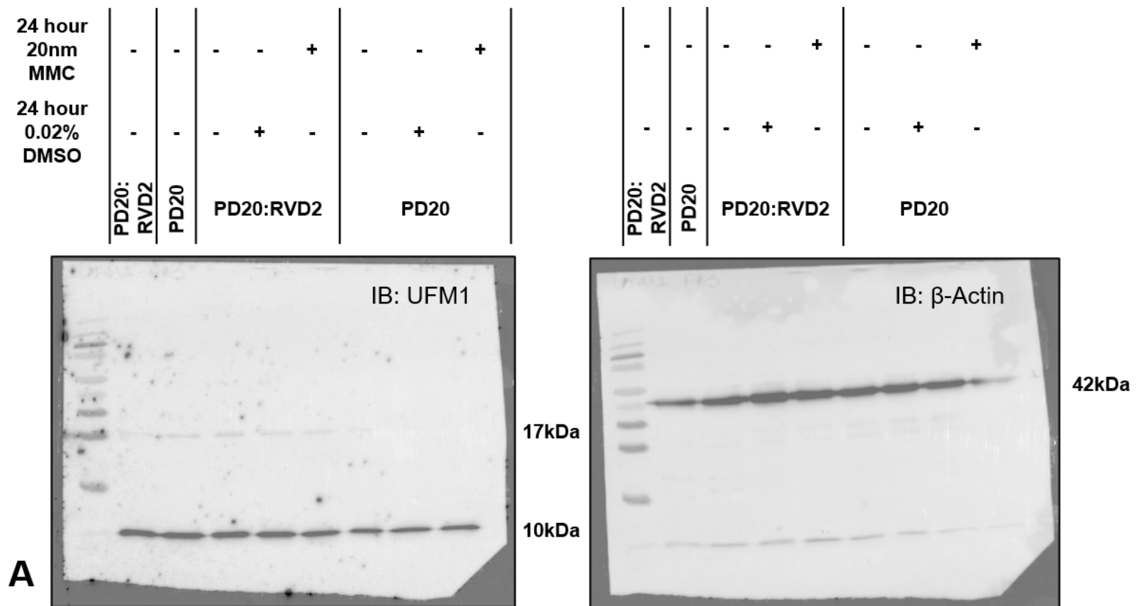
166kDa

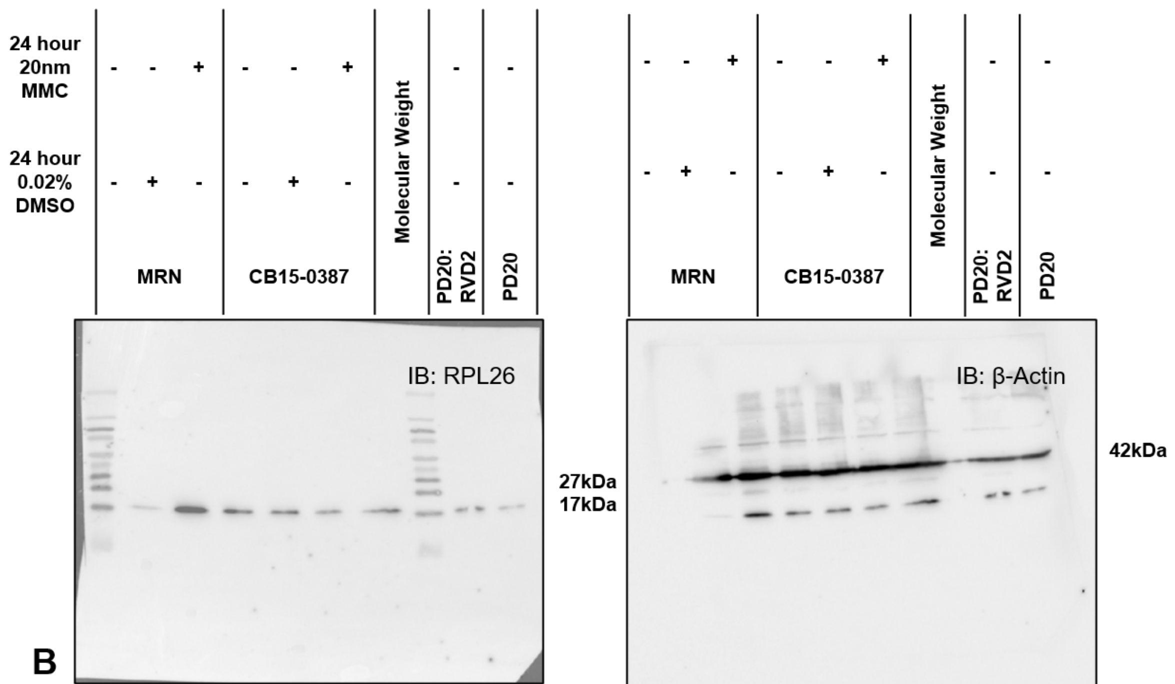
MW	MRN	CB15-0387	PD20: RVD2	PD20	MW	MRN	PD20	CB15-0387	PD20:RVD2



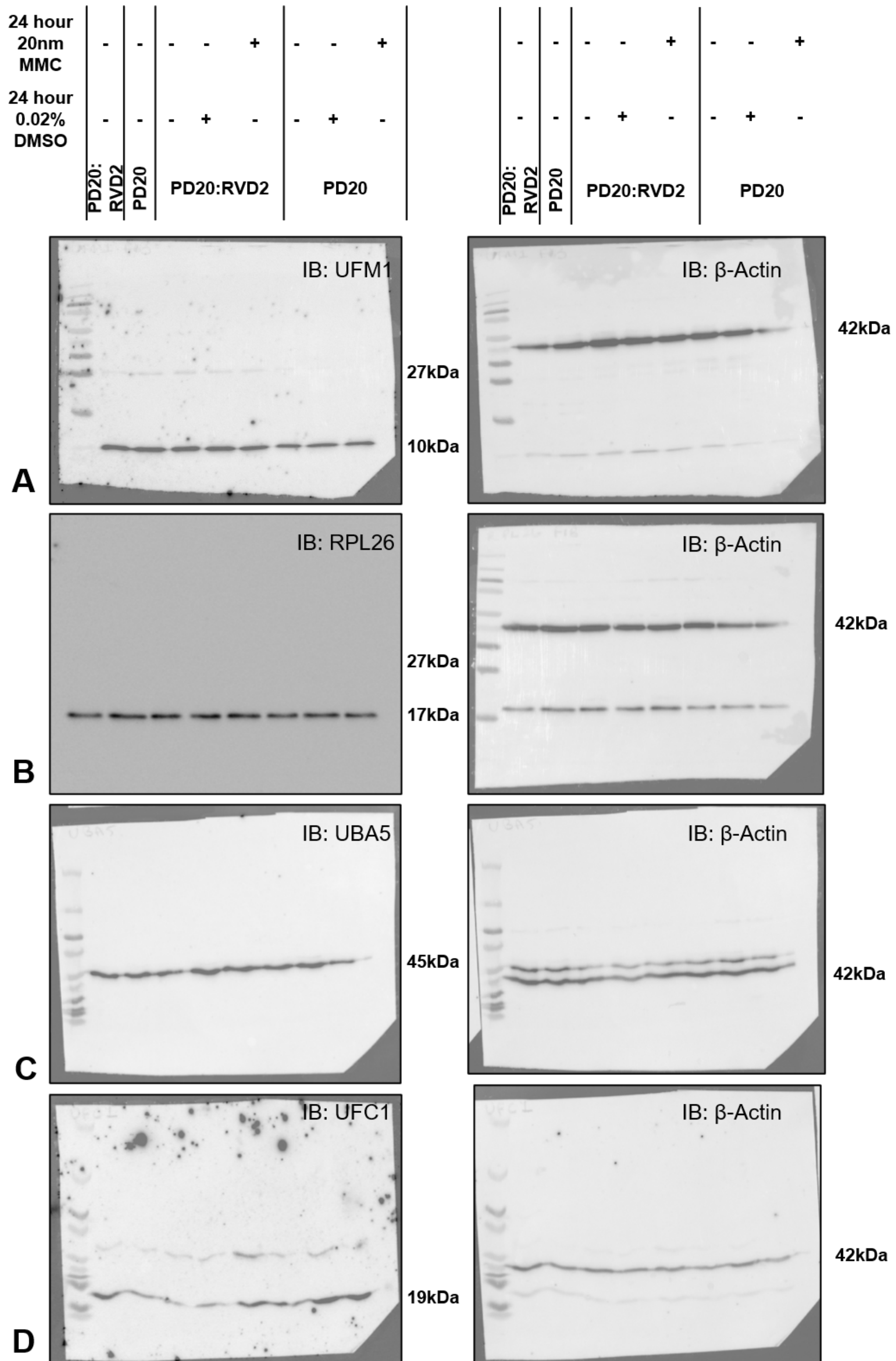
42kDa

Appendix Figure 2 - Full western blot images of Figure 9 and Figure 10.

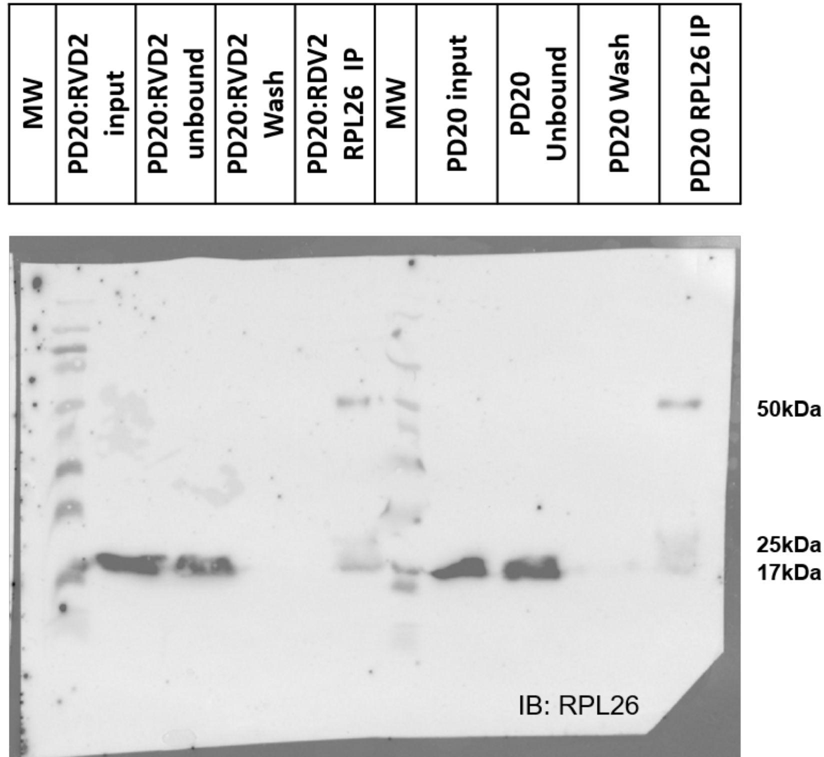




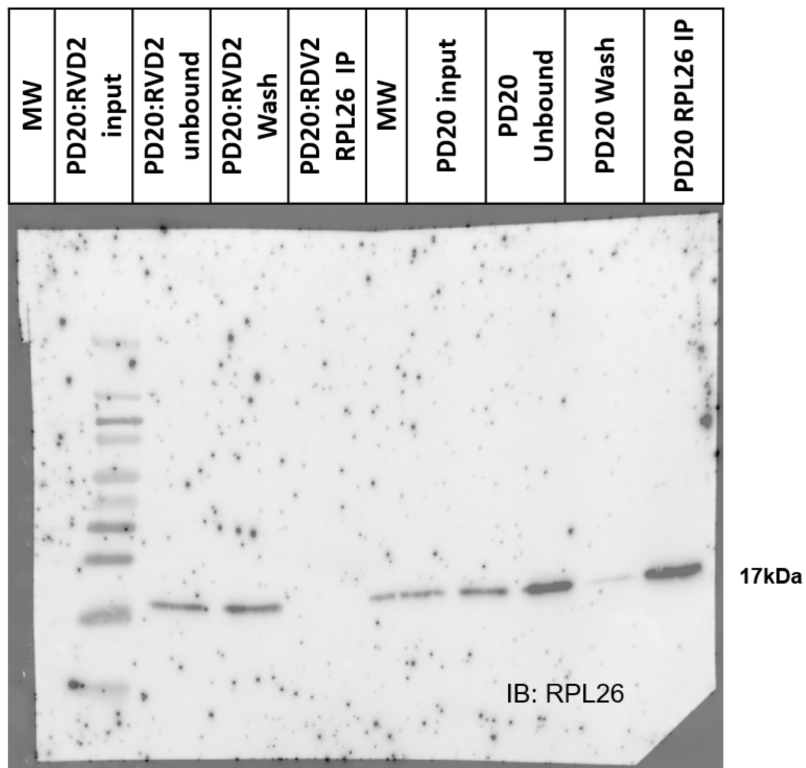
Appendix Figure 3 - Full western blot images of Figure 12.



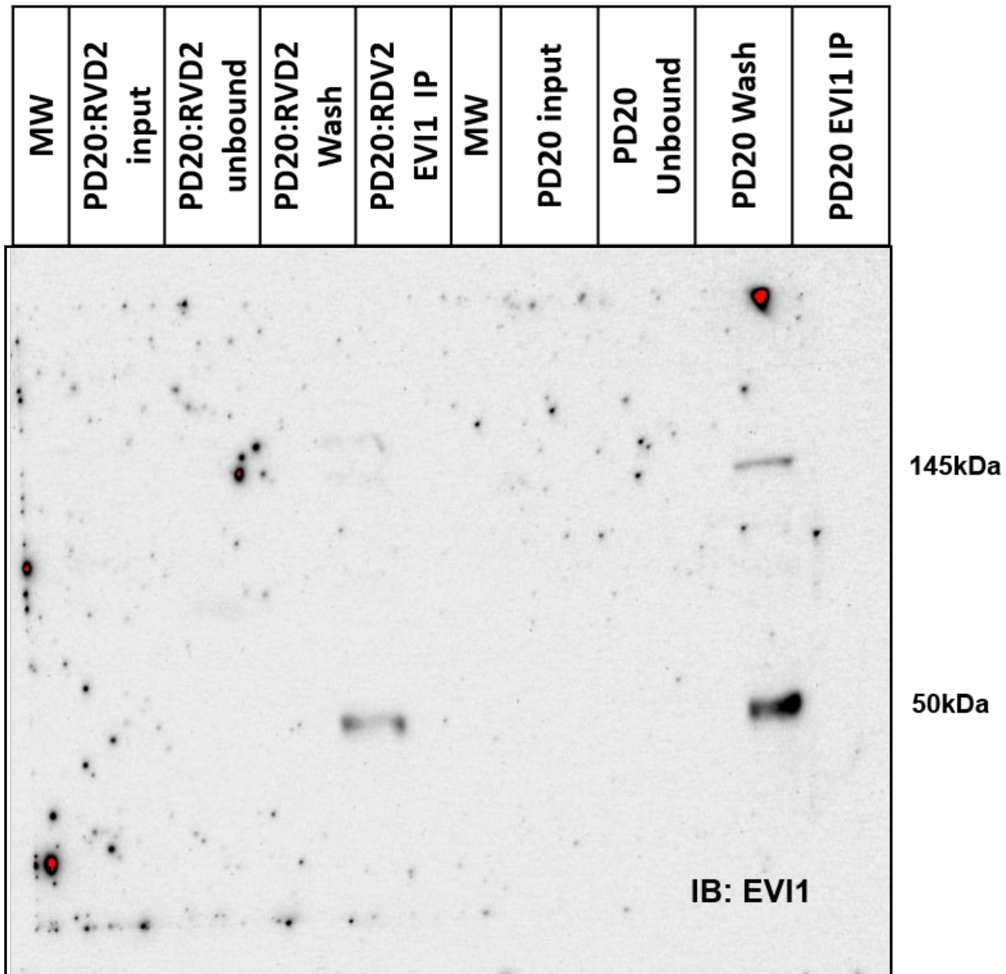
Appendix Figure 4 – Full western blot images of Figure 13.



Appendix Figure 5 - Full western blot image of 200mM NaCl RPL26 IP in Figure 17.



Appendix Figure 6 – Full western blot image of 50mM NaCl RPL26 IP in Figure 18.



Appendix Figure 7 – Full western blot of 200mM NaCl EVI1 IP in Figure 19.