

# MPhil Submission

# Medical School

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**Project Title:**

The Role of FEN1 in Genome Stability and as an Anticancer Target

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Abstract

Flap Endonuclease 1 (FEN1) is a structure-specific nuclease fundamental for DNA replication and repair. FEN1 is attributed as an essential protein for maintaining genome stability due to its activities during cellular DNA metabolism. Dysregulation of FEN1 is linked to carcinogenesis, and haploinsufficiency of FEN1 in mice leads to the rapid onset of tumours. However, it is the overexpression of FEN1 in various cancer types that has resulted in it being postulated as a therapeutic target. Structural studies considering the functions and mechanisms of FEN1 are ongoing and the main aim of my research will be to generate, purify and crystallise untagged human FEN1. It is then postulated that this crystal structure can be used as a drugs target using structure-based drug design.

In this report, I present my findings and discuss the way in which these results impact future work on the topic.

Introduction

Role in Okazaki fragment Maturation

DNA replication is an essential process for cell growth and proliferation. Initiation events occur at multiple points on each chromosome preceding bi-directionally to form replication forks. Both leading and lagging strands are synthesised simultaneously, with the leading strand synthesis (5’-3’) occurring continually in the direction of the replication fork from a single initiator primer. In contrast lagging strand synthesis is semi-discontinuous. Its replication requires the addition of multiple RNA primers, that are then extended to form the newly synthesised DNA (Figure 1).

Early Studies in the Simian Virus 40 (SV40) gave an insight into the mechanisms behind dsDNA replication (Li and Kelly, 1984). In SV40 the viral protein large T antigen, binds the origin of replication and begins unwinding dsDNA. Stimulation of this replication protein by Replication Protein A (RPA) allows the formation of the replication fork (Borowiec et al., 1990). DNA polymerase α/primase (Pol α) acts as an initiator on both the leading and lagging strand, coupling its primer and polymerase activities to synthesise and extend RNA primers (Burgers, 2009, Kunkel and Burgers, 2008, McElhinny et al., 2008). Despite the conservation of a P48 exonuclease domain within Pol α, the protein lacks 3’ nuclease activity, and subsequently these initiator primers have low fidelity, and have to be replaced. Pol α is replaced by Pol ε on the leading strand and on the lagging stand by Pol δ. This polymerase exchange is coordinated by the combined action of PCNA and Replication Factor C (RFC) (Kunkel and Burgers, 2008, Burgers, 2009). Leading strand synthesis only requires Pol α to synthesise a single RNA primer from which Pol ε can continue. On the lagging strand Pol α places frequent RNA primers (7-10 nucleotides) along the DNA, which Pol δ then elongates with 10-20 deoxyribonucleotides (Garg and Burgers, 2005, Zerbe and Kuchta, 2002). These DNA fragments that fill the gaps between RNA primers are termed Okazaki Fragments (Figure 1).

Nucleases within the cell are essential for the processing of RNA primers which must be removed during Okazaki Fragment Maturation. FEN1, Dna2 and RNase H are nucleases implicated in the processing of RNA primers (Bae et al., 2001, Turchi et al., 1994). The proposed methods, by which these nucleases excise RNA primers from DNA, are summarised in figure 2.

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| Figure 1: The major components at the replication fork.  DNA (black lines) is unwound by a DNA helicase to give a leading and lagging strand. DNA is protected from nuclease cleavage by the single stranded binding protein RPA. RNA primers are synthesised by Pol α, initiating DNA synthesis. Pol α is replaced by Pol ε which is responsible for leading strand synthesis and Pol δ for lagging strand synthesis. On the lagging strand Pol δ synthesises DNA fragments (purple) known as Okazaki Fragments in between the RNA primers (pink). These RNA primers are then removed by nucleases including FEN1, DNA2 and RNaseH. Reproduced with permission from (Zheng and Shen, 2011)   |  | | --- | | DNA Pol β  **D**  **A B C** | | Figure 2: Three possible roles of FEN1 in DNA/RNA excision.  A) All but one nucleotide of the RNA primer (pink) is excised by RNase H. The remaining nucleotide is then removed by FEN1. B) The actions of Pol δ lift the RNA primer creating a 5’ flap, which is then recognised and removed by FEN1. C) When the flap generated by POL δ is longer, FEN1 is no longer able to excise it and relies on DNA2 to cleave the flap into a shorter DNA fragment, that FEN1 can then recognise as a substrate. D) Cleavage by FEN1 leaves a ligatable nick which can be sealed by DNA ligase 1. | |

RNase H is a 5’-3’ exonuclease that is expressed at high levels in mammalian cells. The nuclease functions to degrade RNA in RNA-DNA hybrids and has for many years been linked to a role in RNA primer removal (Turchi et al., 1994, Waga et al., 1994). Studies aimed at monitoring RNaseH RNA degradation showed that a 21nt 32P-labelled RNA primer incubated with the nuclease, generated 32P-labelled RNA segments 20 nucleotides in length (Turchi et al., 1994). This suggested that the RNase H specifically cleaves the phosphodiester link one nucleotide upstream from the RNA/DNA junction. This leaves a single RNA nucleotide attached to a DNA fragment on the 5’ flap which is then removed by the exonuclease activity of FEN1 (Nethanel et al., 1992). Although the role of these two enzymes in DNA replication is well established, deletion of one or both genes in yeast was not lethal and did not cause any specific phenotype (Sommers et al., 1995, Frank et al., 1998). This lead to the belief that there was another primary mechanism used to for RNA primer removal.

It has been hypothesised that the actions of the DNA polymerase/PCNA complex is able to displace the downstream Okazaki Fragment during lagging strand synthesis. This displacement leads to the formation of a protruding 5’ flap that can then be processed in two ways, depending on the flap length (figure 2, B and C) (Bambara et al., 1997).

Short Primer Flap Removal

If the RNA flap displaced by DNA Pol δ, is short (7-10 nucleotides), it is recognisable as a FEN1 substrate. The 5’ RNA flap can be bound and cleaved by the exonucleolytic activity of FEN1 creating a ligatable product, which is joined by DNA Ligase 1. In general DNA Pol δ favours the production of RNA-DNA hybrids rather than DNA-DNA hybrids and as such, short flap formation is predominantly seen where only the original RNA primer is displaced (Stith et al., 2008). In addition, the combined presence and action of DNA Pol δ and FEN1 generally keeps flap generation short.

Long Primer Flap Removal

The existence of a long flap pathway was revealed in yeast mutant strains which were lacking the Helicase/Nuclease DNA2 (Budd et al., 1995). Originally it was thought that binding of RPA to long DNA-RNA flaps, allowed recruitment of DNA2, stimulating nuclease activity and cleavage in the DNA region of the flap (Figure 2C). The remaining shortened DNA product is resistant to RPA binding and is processed by FEN1 to give a ligatable product (Bae et al., 2001). Recent studies have suggested that the generation of these flaps is encouraged by the DNA helicase Pif1 (Pike et al., 2009). It is hypothesised that long flaps are simply an elongation of a short flap by the helicase activities of Pif1. The product RNA/DNA flap is then bound by RPA, blocking access and cleavage by FEN1. RPA is able to coordinate the sequential binding of first DNA2 allowing cleavage in the DNA region, followed by FEN1 to create a DNA nick (Bae et al., 2001).

Role in Long-patch Base Excision Repair

FEN1 also plays a critical role in long-patch base excision repair (LPBER) (figure 3). Genome instability is induced by constant exposure to DNA damaging agents, natural cellular DNA breakdown and metabolism by-products such as reactive oxygen species (ROS). In general, a human cell can undergo 1000-10,000 DNA modifications in a single day, all of which require excision and repair to maintain genome fidelity (Drinkwater et al., 1980, Nakamura and Swenberg, 1999). The main method of DNA repair for these lesions is Base Excision Repair, which, can be achieved through the removal of a single nucleotide, Single-Nucleotide Base Excision Repair (SN-BER) or a longer chain, Long-Patch Base Excision Repair (LP-BER) (Sung and Demple, 2006). In both pathways DNA glycosylase, cleaves a glycosidic bond between the phosphodiester backbone of the DNA and the altered base. Cleavage by the DNA glycosylase, leaves an apurinic AP site. The generation of AP sites is not always due to the actions of DNA glycosylase, there are up to 50,00-200,000 non BER related AP sites formed by the spontaneous loss of purines on a daily basis, these too need to be processed by the BER pathway (Nakamura and Swenberg, 1999). AP endonuclease 1 cleaves the phosphodiester backbone of the AP site to leave a 5’ deoxyribose phosphate and a 3’ hydroxyl (Doetsch and Cunningham, 1990). Generally, this initiates SN-BER. DNA Pol β, with both lyase and synthesis function, excises the 5’ deoxyribose phosphate, replacing it with the correct complementary base, producing a nick suitable for ligation by DNA ligase III (Lig III) (Matsumoto and Kim, 1995).

In some instances, DNA Pol β can be post translationally modified, and acylation of this enzyme will inhibit lyase activity (Carter and Parsons, 2016). Additionally, oxidization and reduction of the 5’ deoxyribose phosphate will also prevent DNA Pol β lyase function in SN-BER (Wilson and Barsky, 2001). When this happens, a process very similar to Okazaki Fragment Maturation is initiated. Pol β displaces the downstream 5’ deoxyribose phosphate moiety creating a flap structure. This 5’ flap is then recognised, bound and cleaved by FEN1, leaving a ligatable nick, which is be sealed by DNA ligase I or DNA ligase III (Prasad et al., 1996). FEN1 is believed to stimulate the actions of DNA Pol β and as such they are thought to coordinate both functionally and mechanistically (Liu et al., 2005).

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| DNA Polymerase β  (Synthesis Function)    DNA Polymerase β  (Lyase Function) |
| **Figure 3: Base Excision Repair.**  Both methods of base excision repair are initiated by a DNA Glycosylase which excides the damaged DNA base (shown in red). This leaves an apurinic AP site. An AP endonuclease cleaves the phosphodiester backbone to leave a 5’ deoxyribose phosphate and a 3’ hydroxyl. Ordinarily single nucleotide base excision repair (SNBER) follows, with DNA Polymerase β excising the 5’dRP and then replacing it with the corrected base (shown in blue). This product can then be sealed by DNA Ligase III. Post translational modification to DNA Polymerase β can inhibit lyase activity (represented by the blue Pol β) and subsequently the synthesis function of DNA Polymerase β (represents by the green Pol β) is used to dislodge a 5’ flap that can be recognised by FEN1 as a substrate. This is the initiation of Long-patch base excision repair (LPBER). The cleavage of the substrate by FEN1 leaves a single nucleotide nick that can be sealed by DNA ligase I. |

FEN1 Structure

FEN1 is a 43kDa Mg2+-dependent metalloenzyme (Liu et al., 2004). Comparison of crystallographic structures of FEN1 homologues reveal the conservation of key structural features and suggest a common core architecture, consisting of a central β sheet flanked by a varying number of α helices (Figure 4) (Hwang et al., 1998, Hosfield et al., 1998, Devos et al., 2007, Sakurai et al., 2005, Tsutakawa et al., 2011, Orans et al., 2011, Anstey-Gilbert et al., 2013). The key domains in FEN1 are highlighted in figure 4.

Much of what we know about FEN1 structure was discovered from homologues in bacteria, fungi, plants and viruses. Early indication of a FEN1 structure came from the work done on a bacterial homologue T5FEN. The T5 construct (Ceska et al., 1996) was consistent with the data shown many years later in *Archaeoglobus fulgidus.* These findings suggested that FEN1 preferentially binds to the 3’ flap. This orientates the nuclease into a position that allows precise cleavage 1 nucleotide into the downstream dsDNA (Chapados et al., 2004), producing a nick for ligation. In 2008 preliminary crystallisation studies in human FEN1 confirmed that the 3’ flap was the primary recognition point for FEN1 (Sakurai et al., 2008). It is now proposed that FEN1 specificity is achieved by binding to and bending the DNA to a 100° angle (Tsutakawa et al., 2011). Such a tight bend is only achievable in DNA that contains a flap or a break. FEN1 then binds to the 3’ flap, through a binding pocket created by the α2-α3 loop in the hydrophobic wedge (green). The presence of a 3’ flap pocket and an acid block (red), is a feature conserved among all FENs, giving substrate specificity for a short 3’ flap. This site is only able to enclose a single unpaired nucleotide due to the acid block that inhibits interaction with longer 3’ flaps (Tsutakawa et al., 2011). The recognition of this nucleotide acts as a check point to ensure that the end cleavage point is suitable for ligation.

With the DNA bent and the 3’ flap bound, the β pin, made up of the β6-β7 loop in combination with the α2 helix and the α2-α3 loop form a track to allow correct positioning of the bent DNA aligning the 5’ flap with the active site (Tsutakawa et al., 2011). The 5’ flap is positioned to thread under the cap (pink) (created by helices α4-α5) into the helical gateway (pale blue) and towards the active site. The α2 and α4 helices form the helical gateway and act as guards to the active site, ensuring that only ssDNA can enter, this prevents cleavage of the downstream dsDNA region. For a 5’ flap to pass through this helical arch it must be free of any adducts or branches in the DNA, it must be a “free 5’ flap”. Crystallographic studies in the hFEN1 homologue *Methanococcus jannaschii* (*Mja* Fen1) show the loop formed by the helical gateway produces a hole (8 X 25 Å) large enough to accommodate a single strand of DNA (Hwang et al., 1998). Although highly ordered in some FEN homologues such as the T5FEN (Ceska et al., 1996), other crystal structures such as that of bacteriophage T4 RNase H (Mueser et al., 1996) show this loop to be disordered. This suggests that substrate binding induces a disordered to ordered conformational change.

The active site (figure 4 - orange) is coordinated by divalent metal ions. The β sheet has many conserved acidic residues that form two metal-binding sites (CAT1 and CAT2) in prokaryotic FEN1 and a single CAT1 metal binding site in human FEN1 (figure 5). In eukaryotic FEN1 it has been shown that key residues in the CAT2 site have been substituted, giving a largely hydrophobic region that would not be expected to bind metal ions (Anstey-Gilbert et al., 2013). In human FEN1 seven conserved amino acids Asp34, Asp86, Glu158, Glu160, Asp179, Asp181, and Asp233 (Shen et al., 1996) were found to be within 7Å of a bound Mg2+ ion (Shen et al., 1997). These metal binding sites hold the Mg2+ ions, responsible for the nuclease activity of FEN1(Bhagwat et al., 1997).

At the C-terminus FEN1 is able to bind to the Proliferating Cell Nuclear Antigen (PCNA) (Frank et al., 2001) through a conserved binding region of 9 amino acids QGRLDDFFK (glycine 337 – Lysine 345). This interaction with PCNA is critical for the coordination of FEN1 activity during Okazaki Fragment maturation. (Sporbert et al., 2005).

Substrate specificity is essential and there are 4 primary ways of achieving this. Firstly, the nuclease enforces a sharp bend of 100° on the DNA, this can only be achieved when the substrate either has a flap or a break. Secondly the 3’ flap must only be a single nucleotide long, due to the acid block restricting the binding site created by α2-α3. Third, DNA is forced to pass under the cap before it can enter the active site, only 5’ flaps with free ends can do this. Finally, the active site is guarded by a conserved α2-α4 helical gateway which only allow passage of ssDNA.

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| Figure 4: Structural domains of FEN1.  A - Structural representation of FEN1 bound to DNA. B - The core structural elements essential for FEN1 substrate recognition and cleavage. The acid block (red) is highlighted in red and restricts the binding site created by α2 and α3 only allowing substrates with a single 3’ nucleotide flap. The β6-β7 loop in combination with α2 helix form a track to allow the correct positioning of the DNA to align the 5’ flap with the active site. Before reaching the active site (orange) substrates must pass through the Cap (pink) formed by α4 and α5, this prevents the passage of any 5’ flaps with adducts. As the DNA approaches the active site it will pass through the helical gateway (blue). Only single stranded DNA can fit through the gateway and proceed to the active site. (Tsutakawa et al., 2011). |

Nuclease Activity

Enzymatic activity in FEN1 requires the presence of divalent ions occupying the CAT1/CAT2 sites (Lee and Wilson, 1999, Orans et al., 2011), this is emphasised by the conservation of the seven or eight acidic aspartate and glutamate residues in the active site that form the metal binding sites as shown in figure 6 (Shen et al., 1996). Studies in human FEN nuclease EXO1 show function is optimal in the presence of Mg2+ ions. Substitution of Mg2+ with other cations such as Ca2+ or Ba2+ result in a loss of nuclease activity and substitution of Mg2+ for Mn2+ in FEN1 showed comparable activity levels of <5% (Orans et al., 2011).

Studies looking into the specific functions of metal binding in these sites, have produced conflicting theories. Mutational analysis removing two of the conserved aspartate residues in the CAT2 region of T5FEN, showed that this site was not relevant for catalysis and instead was required for substrate binding (Tomlinson et al., 2011). This was supported by similar mutations in T4 RNase H (Bhagwat et al., 1997). In contrast site mutations within the CAT2 region of DNA Polymerase 1 5’ nuclease in *E.Coli* and *Myobacterium Tuberculosis* caused loss of enzymatic activity (Xu et al., 1997, Mizrahi and Huberts, 1996). CAT1 site binding is less controversial, in most studies disruption of metal binding to this site has caused loss of nuclease activity. In the bacteriophage T5 homologue (T5FEN) occupation of the CAT1 site is essential for nuclease activity and flap cleavage (Feng et al., 2004).

It is debated how these cations facilitate enzyme activity. Protein nucleases involved in RNA/DNA repair and degradation are commonly proposed to catalyse hydrolysis of phosphodiester bonds using a “two-metal-ion-mechanism”. For this to occur in FEN1 the two magnesium ions must be within 4Å of each other. In most FEN1 crystal structures the position of M1 is similarly positioned whereas the position of M2 varies. The currently available human FEN1 and human EXO1 structures support the two-metal ion theory, showing metal ions 4Å apart, coordinating phosphate groups (Tsutakawa et al., 2011, Orans et al., 2011). However, these FEN1 and EXO1 structures were solved without Mg2+ ions (Sm3+ in hFEN1 and Ba2+ and Mn2+ in hExo1) and as such are not biologically relevant. When studying the prokaryotic FEN structures the Mg2+ ions are 8Å apart (Dupureur, 2010), which would mean that coordination between them is not possible.

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| Figure 5: FEN1 Active site comparisons.  a) Direct comparison between the human (green), bacterial (purple and blue), and archaeal (brown) FEN1 active sites. Bacterial (b and c), human (d) and archaeal (e). Reproduced with permission from (Syson et al., 2008) |

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| Human vs mj FEN1    T5 VS T4 FEN1 |

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| Figure 6: Protein Sequence Alignment of human / mj FEN1 and T5 / T4 FEN1. Highlighted in yellow are the conserved catalytic amino acid residues D34, D86, E158, E160, D179, D181 and D233 in human FEN1 and D27, D80, E152, E154, D173, D175 and D224 in mj FEN1. D26, D68, E128, D130, D153, D155, D201 and D204 in T5 FEN1 and D19, D71, E130, D132, D155, D157 D197 and D200 in T4 FEN1. |

Tracking Vs Threading

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| During Okazaki fragment maturation and LPBER, FEN1 binds to and cleaves 5’-flaps. Flaps containing Biotin−streptavadin adducts (Murante et al., 1995), platinum adducts or branches structures (Bornarth et al., 1999) prevent FEN1 cleavage. These early results led to the suggestion that FEN1 used a tracking mechanism (figure 7) which requires a free 5’ flap for the nuclease to attach to before traversing its way down to the site of endonucleolytic cleavage. (Murante et al., 1995, Kao et al., 2004, Bornarth et al., 1999). The tracking theory was popular as it was believed to play a role in protecting the genome. If FEN1 was required to bind to the 5’ flap and move down it to the site of cleavage then, then single stranded fragments between okazaki fragments would be safe from undesired nucleolytic activity. However more recent studies have shown that FEN1 is able to bind to substrates with 5’ flap modifications (Gloor et al., 2010) suggesting an alternative mechanism. This newly proposed method of cleavage is termed threading (figure 7) and proposes that FEN1 first binds to the base of dsDNA, causing it to bend to a 100° angle that allows the 5’ flap to thread under the cap and into the helical arch (Tsutakawa et al., 2011). There is biochemical evidence to suggest that specific amino acid interactions with the dsDNA ensure binding of the protein to the 5’ flap does not occur (Gloor et al., 2010). Where it was previously believed that a free 5’ flap was needed for binding it has now been shown that the 5’ flap must be free of modifications to allow threading into the arch.   |  | | --- | |  | | Figure 7: FEN1 tracking vs Threading.  Schematic representation of FEN1 tracking and threading its substrate. In the tracking mechanism, FEN1 binds the end of the 5’ flap and transverses its way down to the site of cleavage. However, more recent studies have shown that FEN1 can still bind substrates with 5’ flap modifications suggesting FEN1 binds the base of the dsDNA and the 5’ flap threads through the active site allowing cleavage. | |

A recent publication reported three crystal structures of the bacteriophage T5 flap endonuclease in complex with DNA substrates. These structures which are presented at resolutions pf 1.9-2.2 Å show single stranded DNA threading through the helical arch of the enzyme (figure 8) (AlMalki et al., 2016) .

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| Figure 8: The FEN1 catalytic cycle.  The DNA substrate binds to a disordered FEN1 (top centre). Single stranded DNA is then threaded through the helical arch (top right). FEN1 undergoes a disordered to ordered conformational change of the helical arch after DNA threading (bottom right). The DNA substrate is cleaved, and the product released (bottom centre). |

How FEN1 cleaves DNA had been the subject of debate for many years with some suggesting that the threading mechanism would be thermodynamically unfavourable (Xu et al., 2001). These recent studies have led to an improved understanding of the processing of FEN1 substrates and revealed a FEN1 mutant (D153K) that is catalytically inactive. This D153K FEN1 mutant is of specific interest to myself as I can use mutations that map to the same residue in the human FEN1 (D179K) to get a human crystal structure bound to a FEN1 substrate. All previous structures of the human FEN1 have been bound to a product as FEN1 cleavage has already occurred. The substitution of Aspartic Acid to Lysine in the active site has however allowed binding but not cleavage of the substrate and therefore given a clear understanding of FEN1: substrate binding (figure 8). Aspartic Acid is a large bulky negatively charged amino acid and the substitution to Lysine, a smaller positively charged amino acid, will likely alter the active site binding with the negatively charged phosphate backbone of DNA. Other amino acid substitutions to consider would be a smaller amino acid, for example glycine or serine, as these would provide a structural difference in the active site. Alternatively, substitution to arginine may prove interesting; differing only in that it contains an amino group in place of one of the oxygens found in aspartate and thus also lacks a negative charge.

FEN1 and Cancer

Both Okazaki fragment maturation and LBPER, are essential processes for maintaining genome stability. Dysregulation of these processes can lead to DNA strand breaks, halting of DNA replication/repair and genome instability. For these reasons we postulate that differentiation in function or expression levels of key enzymes within these processes, such as FEN1, can give mutator phenotypes and contribute to cancer initiation or progression. Severe mutations or deletions in the genes responsible for these processes, would rarely be seen in humans as this would most likely lead to cell death. Therefore, it is suggested that minor mutations that cause subtle changes to function are existent in the population and may contribute to an increased chance of early onset cancer; such mutations in FEN1 will be discussed below. In addition to mutations in these key enzymes, cancer tumour profiles often show overexpression of essential proteins involved in DNA replication and repair. As we will see this is indeed the case for FEN1. As accelerated cell growth and proliferation is a hallmark of cancer, deregulation of replication/repair enzymes such as FEN1 could be seen to facilitate this increased proliferation and thus inhibition of these enzymes is considered a valid chemotherapeutic target.

FEN1 Mutants

While it has been shown that FEN1 is not essential for the cell viability of *S.cerevisiae,* aberrations to the protein that alter activity do effect cell growth and proliferation, usually resulting in death. In *S.cerevisiae* experiments that mutate the FEN1 homologue RAD27 giving a null mutant of the nuclease cause increased sensitivity to the DNA alkylating agent methylmethane sulfonate (Reagan et al., 1995). Mutation to the FEN1 PCNA binding motif (337)QGRLDDFFK(345) in mice, disrupts binding to PCNA and as a result FEN1 is unable to localize to sites of replication, compromising Okazaki Fragment maturation (Zheng et al., 2007a). This binding motif mutant (F343A/F344A-FFAA) still retains nuclease activity, however FFAA/FFAA mutants, displayed slow growth and died immediately after birth. Surprisingly the FFAA mutants were able to carry out Okazaki Fragment maturation, but they accumulated unligated nicks in the DNA, which collapsed replication forks in the subsequent round of replication. (Zheng et al., 2007a). Further work by Zheng identified human FEN1 mutants lacking exonuclease activity. When introduced to mice the E160D mutation reduced exonuclease activity to 10% (Zheng et al., 2007a). Interestingly these mutants retained endonuclease activity and the mice grew and developed, retaining near normal DNA replication and proliferation rates. However, with a lack of exonuclease activity the FEN1 loses the ability to edit out Pol α errors and subsequently high levels of base substitution were observed in mutant mice, predisposing them to early onset lung cancer (Zheng et al., 2007a).

The evidence put forward by Zheng would suggest that mutations to FEN1 could contribute to cancer in two separate ways. Firstly, mutants that remove endonuclease activity and as such prevent appropriate Okazaki fragment maturation, will lead to double strand breaks in DNA, genome instability and cancer progression associated with chromosomal irregularities. Secondly, mutants that effect exonuclease activity and the ability of FEN1 to excise misincorporated DNA bases, would lead to a mutator phenotype.

There are 51 mutations that have been noted in the human FEN1 gene as shown in the histogram in figure 9. Point mutations to the FEN1 gene have been identified in samples across 15 tumour types including, liver, lung, cervical, breast and prostate. However, these point mutations were present in less than 1% of any of the sample cohorts, with overexpression of FEN1 in the same samples being much more prominent. For example, of the 1104 breast cancer samples tested, 78 showed overexpression of FEN1 (7.07%) whereas only 3 samples contained point mutations (0.11%) (COSMIC, 2018).

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| Figure 9: Gene view histogram of mutations across FEN1.  These mutations are displayed at the amino acid level across the full length of the gene (COSMIC, 2018) |

Figure 10 shows an overview of the types of mutations seen in the FEN1 gene. Over 62% are missense mutations, however none of these were at active site amino acids and only one was seen in the PCNA binding motif (COSMIC, 2018). The R339H mutation in the PCNA binding domain was found in a colon adenocarcinoma sample and as expected disrupted FEN1 localisation (ICGC Data Portal, 2018).

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| Figure 10: An overview of the types of mutations observed in FEN1.  There are no deletion or insertion mutations noted, all mutations observed are substitutions, the majority of which are missense. (COSMIC, 2018) |

Fen1 Overexpression

Studies have suggested that knock out of FEN1 activity and haploinsufficiency of the nuclease in cells may lead to cancer progression. For this reason, FEN1 is seen as a tumour suppressor. However, FEN1 expression is proliferation dependent and usually expression of the enzyme is negligible in non-proliferating cells (Warbrick et al., 1998). Conversely in cancer cells, core mechanisms within the cell are deregulated to allow accelerated proliferation and growth. As FEN1 is central to DNA replication and repair, its overexpression in most of cancer cell types is unsurprising.

Singh *et al* compared the expression levels of FEN1 to that seen in normal tissue and showed that FEN1 is overexpressed in many cancers, including breast, uterine, kidney, lung and pancreatic cancer (Figure 11) (Singh et al., 2008). Often this overexpression was a result of increased mRNA, most likely due to the hypomethylation of the FEN1 promoter (Singh et al., 2008). There is now growing belief that FEN1 expression is not only involved in the development of cancer, but its overexpression may actively help cancer progression (Zheng et al., 2007b).

FEN1 and Lung Cancer

Lung cancer is one of the most common cancers in the world, with an average 5-year survival rate of 15%. The disease has little to no symptoms in the early stages making early prognosis hard to achieve. As a result lung cancer is the most prevalent cause of cancer related death worldwide, killing over 1.5 million people every year since 2012 (WHO, 2015). With few therapeutic options and such a poor survival rate, overexpressed FEN1 is now an attractive target for lung cancer treatment.

FEN1 expression in normal tissue, was compared to cancerous tissue from the same patient. 241 individual tumour samples and the corresponding normal tissue were taken from the same patient and hFEN1 was used to synthesise 32P-labelled cDNA probes. The samples were then hybridized with the FEN1 probes and exposed to phosphorimaging screen, scanned with a phosphorimager and the results quantified with Image Quant 1.2 software. After quantification, the results showed consistent overexpression of FEN1 across a range of cancer types (figure 11). Many of the differences in expression were statistically relevant, with lung cancer tissue showing a 1.9-fold increase in FEN1 (Singh et al., 2008).

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| Figure 11: FEN1 cDNA expression in different cancer types**.**  Expression of FEN1 cDNA in matched normal and tumour tissues was achieved using a Cancer Profiling Array containing cDNA samples from 241 paired human tumours (grey) and normal (white) tissue from individual patients. A statistically relevant increase in FEN1 cDNA expression was seen in lung cancer (1.9 fold increase, P<0.0066). Reproduced with permission from (Singh et al., 2008). |

Overexpression of FEN1 in lung cancer was also shown in Nikolova *et al*., who’s study elucidated the level of expression of FEN1 protein in cancer of testis, lung and brain. FEN1 protein expression was studied by Western blot analysis in specimens of tumour tissues compared with the normal tissue from the same patient. The expression levels were quantified using the loading control ERK2, and showed that 4/4 samples were overexpressing FEN1 (Nikolova et al., 2009).

A recent publication from He et al showed FEN1 mRNA expression levels in lung cancer tissues was significantly higher than that in normal tissues (Figure 12 A). This was further confirmed by immunohistochemistry (IHC) assays comparing the FEN1 protein expression level on normal and lung cancer samples from surgical treatment (Figure 12 B). FEN1 overexpression in lung cancer cell lines was also shown by western blotting (figure 13). Lung cancer cell lines (A549, H1299, and H460) displayed noticeably higher FEN1 expression level than the normal lung cell line (HELF). Despite showing FEN1 overexpression in lung cancer cell lines, it was still not known if this increased expression correlated with increased tumour progression or a poor prognosis.

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| Figure 12: FEN1 overexpression in lung cancer cells.  (A) FEN1 expression analysis based on TCGA dataset showed that FEN1 mRNA levels were higher in lung cancer tissue than in normal tissue (\*P < 0.01 vs control group). (B) FEN1 displayed significantly stronger staining (brown) in tumour specimens from clinical patients than from healthy counterparts. Immunohistochemistry was performed on formalin-fixed and paraffin-embedded tissues using antibodies against FEN1. Original magnification, × 400. Scale bars, 250 μm. Reproduced from (He et al., 2017) |

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| Figure 13: FEN1 protein expression levels in different cell lines.  Lung cancer cell lines (A549, H1299, and H460) displayed significantly higher FEN1 expression level than the normal lung cell line (HELF). Reproduced from (He et al., 2017) |

FEN1 and Prostate Cancer

43,436 men were diagnosed with prostate cancer in 2012, making it the second most common cancer amongst men in the UK (CRUK). In the same year 10,837 patients died. Worldwide it was estimated that 1.1 million men were diagnosed in 2012(CRUK). Advances in prostate cancer detection such as PSA testing and the Gleason score have meant that survival rates have tripled in the last 40 years in the UK (CRUK). Although there have been many improvements in diagnosis and treatment, there is still no way of assessing if a tumour is clinically relevant, or accurately predict a patient’s outcome (Hernandez and Thompson, 2004), this leads to over treatment of many men. As a result, an effort is being made to find new diagnostic markers. Further treatment options for aggressive disease are limited.

FEN1 mutations in prostate cancer are yet to be explored, however overexpression of FEN1 in aggressive tumour types is well documented in the literature. An oligonucleotide microarray on the most aggressive clone of prostate cancer currently available CL 1.1, identified FEN1 as overexpressed in comparison to non-tumour clones. Figure 14 shows the mean expression of FEN1 protein in prostate cancer, benign prostatic hyperplasia (a non-cancerous enlargement of the prostate), prostatic intraepithelial neoplasia (abnormality of the prostate glands, that usually precedes prostate adenocarcinoma) and normal prostate epithelium (Lam et al., 2006). Expression was assessed semi quantitively from immunohistochemical staining using a monoclonal anti-FEN1 antibody (NCL- Fen-1, Novocastra, Newcastle-upon-tyne, UK, 13 μg / ml at final dilution) FEN1 expression was significantly higher in cancer (36.7%) than in normal tissue (13.2%). Intriguingly FEN1 expression in prostatic intraepithelial neoplasia (15.4%) was higher than in normal prostate epithelium, suggesting that FEN1 expression has potential to be used as a diagnostic tool to ensure the disease is caught in its early stage.

The FEN1 protein expression from the immunohistochemical staining was then cross matched with the Gleason score for each tumour sample showing a significant correlation between FEN1 and Gleason score (Lam et al., 2006), further suggesting a potential role for FEN1 as a prostate cancer biomarker. Figure 15 depicts FEN1 protein expression across the full Gleason grading scale; it shows that between grades 4 and 8 there is a consistent increase in FEN1 expression however scores 3 and 9 stray from this trend. Although the trend does not perfectly match all grades, the correlation between FEN1 protein expression and Gleason score is statistically significant p=0.002 (Lam et al., 2006). In addition to the findings from Lam *et al*, in 2012 nuclear staining of Prostate cancer (PC) and castration-resistant prostate cancer (CRPC) samples showed that there was a significantly high staining frequency for FEN1 (Urbanucci et al., 2012). More staining was seen in CRPC samples than PC samples and higher levels of FEN1 overexpression could be correlated with a poor prognosis in prostatectomy treated patients (Urbanucci et al., 2012). As FEN1 is clearly overexpressed in prostate cancer and is often associated with poor prognosis and a high Gleason score, it is likely to be a putative drug target.

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| Figure 14: FEN1 protein expression in prostate cancer**.**  Mean number of cells that stained positive for FEN1 in prostate cancer (CaP), benign prostatic hyperplasia (BPH), normal prostate epithelium and prostatic intraepithelial neoplasia (PIN). Reproduced with permission from (Lam et al., 2006). |
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| Figure 15: FEN1 protein expression (%) in each Gleason score.  The median is represented by the middle line, with the end lines attached to the box giving the upper and lower values. The number of tumour spots analysed is depicted by the width of the box and the top and bottom of the boxes show the median of the upper and lower halves of the data. Reproduced with permission from (Lam et al., 2006). |

FEN1 Inhibitors

Due to its overexpression in various cancer types and its role as a propagator in cancer cell growth there have been many attempts to develop FEN1 inhibitors. Currently there are no lead FEN1 inhibitor compounds available, as most of the small organic compounds identified to inhibit FEN1 also having off target effects causing serious issues with metabolic processing (Tan et al., 2006). Many methods have been used to identify FEN1 inhibitors from small molecule screens to high-throughput assays based on a change in fluorescence polarisation of a labelled FEN1: DNA substrate (figure 16) The latter was a project undertaken by AstraZeneca, where they used this high-throughput assay to screen against a library of 850,000 compounds (McWhirter et al., 2013). Although hits were found Astra Zeneca eventually dropped the project, as no inhibitors were viable for clinical trials.

It is still accepted that inhibition of FEN1 could still be a valid cancer therapeutic both as a monotherapy and as a chemosensitiser. However, new inhibitors are needed that are more specific and more effective. With the recent findings that the FEN1 D179K mutant does not have catalytic ability, this opens a new novel method of drug development. If a substrate: FEN1 compound can be crystallised, then this compound can potentially be used to screen for potential inhibitors. Molecules that can specifically bind to FEN1 and its substrate might be able to lock the enzyme in this conformation rendering it inactive. Furthermore, the extra specificity needed to bind the compound as a whole could prevent some of the off-target effects that have been found previously.

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| Figure 16: Astra Zeneca Assay Schematic.  Compounds were screened for their ability to inhibit FEN1. When incubated with uninhibited FEN1 the flap structure would be cleaved and give a fluorescent product with a reduced mass. If, however the FEN1 was inhibited the mass of the fluorescent substrate would remain the same. |

Hypothesis

We hypothesise that a human FEN1: substrate-DNA crystal structure can be obtained with a D179K active site mutant designed to permit binding but not catalysis

We hypothesise that Phenotypic characterisation of the effects of expression of the D179K FEN1 mutant in human cells can be achieved with new, more specific FEN1 inhibitors

We hypothesise the overexpression of FEN1 in cancer cells facilitates chemoresistance by reducing the impact of alkylating agents. As such we believe that specific inhibition of FEN1 will increase sensitivity of cancer cells to chemotherapy.

Aims

To overexpress and purify recombinant untagged human FEN1.

To produce the D179K active site mutant that can bind DNA but is catalytically inert in the presence of magnesium.

To crystallisation a substrate DNA-FEN1 structure, using WT and D179K human FEN1 for use as a therapeutic drug target.

Materials

Plasmids

All plasmids were obtained from Sarah Oates a previous member of the Sayer lab. (Oates, 2016)

Table 1: Plasmids used for the overexpression of WT and D179K FEN1.

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| Name | Antibiotic Resistance | Mammalian (M) / Bacterial (B) Expression | External Sequencing Primers |
| WT FEN1 Pet-21a+ | Ampicillin | B | T7 Forward & Reverse |
| D179K FEN1 Pet21-a+ | Ampicillin | B | T7 Forward & Reverse |
| WT FEN1 pDEST 12.2 | Ampicillin | M | SP6 Forward & T7 Reverse |
| D179K FEN1 pDEST 12.2 | Ampicillin | M | SP6 Forward & T7 Reverse |

The plasmid used for all bacterial work is the pET-21a+ plasmid (Novagen). As shown in figure 17 it contains an antibiotic selection marker that encodes resistance to ampicillin, allowing for selection of E.coli cells that contain the plasmid.

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| Figure 17: pET-21a(+) vector Sequence.  The pET-21a(+) contains a T7 forward and reverse promoter for sequencing and also encodes ampicillin resistance. (SnapGene, 2018) |

The plasmid used for all mammalian work is the pDEST 12.2 plasmid. Figure 18 shows the pDEST 12.2 vector map, containing ampicillin resistance and SP6 forward and T7 reverse promoters. DNA from the entry clone will occupy the region between nucleotides 738 and 2419 (Invotrogen, 2018).

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| Figure 18: Map of the pDEST 12.2 vector.  The pDEST 12.2 vector encode SP6 forward and T7 reverse promoters for sequencing and also encodes ampicillin resistance (Invotrogen, 2018) |

The sequences covered in the plasmids included the full-length WT FEN1 as seen in figure 6 and a c-terminal truncated WT and D179K FEN1 mutants that compromised of amino acids 1-336. This C-terminal truncation was used to aid crystallography of the protein, as the c-terminal amino acid residues encode the flexible PCNA binding domain which is thought to be disordered and disordered regions do not form a fixed structure for crystallisation (Finger et al., 2012; Le Gall et al., 2007).

Protein overexpression, purification and analysis

**Kanamycin** sulphate stock 50 mg/ml

**Ampicillin** sodium salt stock 100 mg/ml

**Lysogeny Broth (LB) media:** Tryptone (10 g), Yeast Extract (5 g) and NaCl (10 g) were added to 1 L of water and autoclaved.

**5YT Media:** Tryptone (40 g), Yeast extract (25 g) and NaCl (5 g) were added to 1 L of water and autoclaved.

**Agar Plates:** A 300ml solution consists of 4.5 g 2% (w/v) Agar and 7.5 g LB. The solution is autoclaved and left in a water bath at 60°C prior to use. Glucose (1% w/v) and antibiotics are added prior to pouring, when the agar had been cooled below 60°C.

**Lysis Buffer:** 50 mM Tris pH8, 2 mM EDTA, 200 mM NaCl, 5% (v/v) glycerol

**Phosphate Buffer**: 20 mM K2HPO4, 20 mM KH2PO4, 1 mM DTT, 2 mM EDTA and 5% glycerol

**Resolving Gel (10%):** deionised water (4.5 ml), 500mM Tris Bicine pH 8.3 (2 ml), 10% (w/v) SDS (100 μL), 30% (w/v) acrylamide (3.3 ml), ammonium persulfate (50 μl), TEMED (16 μL).

**Stacking Gel:** deionised water (1 ml), 250 mM TrisHCl pH 6.9 (1.5 ml), 10% (w/v) SDS (30 μl), 30% (w/v) acrylamide (500 μl), ammonium persulfate (20 μl), TEMED (10 μl).

**2x Protein Loading Dye:** 0.2% SDS, 2.5% glycerol, 75 mM tris-HCl, 0.5% bromophenol blue (BDH), 5 mM EDTA and DTT

**Coomassie Blue Stain:** 40% (v/v) methanol, 10% (v/v) acetic acid, 0.2% w/v coomassie brilliant blue

**De-staining Solution:** 20% (v/v) methanol and 10% (v/v) acetic acid

Mammalian Tissue Culture

**Foetal calf serum:** Supplied by Seralab and stored at -20°C until needed.

**Dulbeccos modified eagles medium (DMEM):** High glucose (4.5 g/L) DMEM containing L-glutamine supplied by Lonza.

**Trypsin:** Containing 0.2 g/L Versene and 0.5 g/L Trypsin, supplied by Lonza.

Methods

Electrophoretic Methods

Agarose Gel Electrophoresis

1% Agarose gels were prepared by boiling Agarose powder in TAE buffer. After cooling Ethidium Bromide (0.5 μg/ml) was added and swirled to mix. The solution was poured into a cast and a comb inserted to create an appropriate number of wells. Once set the comb in removed and the gel placed in a tank containing 1 x TAE. Samples are loaded and run at 60V before being visualised in a UV transilluminator.

Denaturing SDS-PAGE

SDS-PAGE was used to visualise proteins that when denatured are separated by size.

FEN1 proteins were analysed on 10% resolving gels which were prepared as described in the materials section. After the resolving gel was poured into the cast and left to set, the stacking gel was poured on top and a comb inserted to create sample wells. Samples were mixed 1:1 with loading buffer, then boiled on a hot block at 95°C for 3 minutes and spun down in a micro centrifuge for 10 seconds. Protein samples were loaded into the wells in the stacking gel and run at 35 mA for 1 hour. As the proteins pass through the resolving gel they are separated by size with heavier proteins travelling slower. The gel was stained in coomassie brilliant blue solution and excess dye removed using de-stain allowing the proteins to be visualised.

Western Blotting

Western blots enable us to measure the relative amounts of protein in different samples.

10 x transfer buffer – 30.3 g Tris, 144 g glycine, distilled water to 1000 ml.

An SDS-PAGE gel is soaked in 1 x transfer buffer (10% 10 x transfer buffer, 20% Methanol, 70% ddH2O) for 15 minutes. The gel is placed on top of a nitrocellulose square and placed between filter paper. The gel is transferred onto the nitrocellulose at 30V for 1 hour in a tank containing 1 x transfer buffer. Once finished the nitrocellulose is removed from the cassette and the membrane is blocked with 5% milk in PBS for 1 hour. Appropriate antibody is then diluted in 5% milk in PBS-0.05% Tween, 5ml is added to the nitrocellulose and left overnight at 4°C. The membrane is washed for 10 minutes, three times in PBS-Tween, before adding a secondary antibody which has a specific enzyme e.g. horseradish peroxidase attached to it for visualisation. The secondary antibody is added to 5% milk in PBS-Tween and incubated at room temperature with the membrane for 1 hour. Visualisation of the secondary antibody and therefore the protein of interest is achieved by incubating the membrane with a chemoluminescence reagent (ECL). A 1:1 ratio of part A and part B are mixed to a total volume of 2 ml which is then added to the nitrocellulose and left to incubate for 1 minute, before exposing the membrane to X-ray film.

Polymerase Chain Reaction

PCR mixes were set up using the constituents and concentrations in table 2 and run on the PCR cycle shown in table 3.

Table 2: Components and Volumes for PCR Mutagenesis

PCR was made to a final volume of 50µl using sterilised H2O

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| Component | Final Concentration |
| 2 X KAPA Hot Start Ready Mix | 1 X |
| Forward Primer | 0.3 μm |
| Reverse Primer | 0.3 μm |
| Template DNA | 40 ng |

Table 3: PCR Cycling Protocol

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| --- | --- | --- | --- |
| Cycle Section | Temperature (°C) | Time (Seconds) | Number of Cycles |
| Initial Denaturisation | 95 | 180 | 1 |
| Denaturisation | 98 | 20 | 20 |
| Annealing | 65 | 15 | 20 |
| Extension | 72 | 15 | 20 |
| Final Extension | 72 | 60 | 1 |

Transformation of FEN1 plasmid into Competent Cells

Plasmid DNA (1 µl), was added to competent XL10 cells (50 µl) for bacterial expression or DH5α (50 μl) cells for mammalian expression and left on ice for 30 minutes. Samples incubated in a water bath heated to 42°C for 45 seconds, then returned to ice for 2 minutes. Cells were plated on to ampicillin selective plates and incubated overnight at 37°C.

Plasmid Extraction

Miniprep and Maxiprep kits were used from Quiagen and Sigma Aldrich following the manufacturer spin protocols.

Protein Overexpression and Purification

WT and D179K FEN1 protein were overexpress in *E.coli* using IPTG induction Purification was achieved using a variety of methods including Ion exchange chromatography and affinity chromatography. Two purification methods have been designed, one for crystallography purposes and the other for biochemical assay. Both are detailed below.

Protein Overexpression

A single colony from a newly streaked agar plate was picked and inoculated in to 5 ml of 5YT containing 0.1 mg/ml ampicillin and 0.1% glucose. The media was left shaking for 8 hours at 37°C. This was then added to 500 ml of 5YT containing 0.1 mg/ml ampicillin and 0.1% glucose and left shaking overnight at 37°C. 3L of media (5YT, 0.1% glucose and 0.1 mg/ml ampicillin) was inoculated with the overnight 500 ml culture at 37°C until the reach an A600 of 0.6. At this stage 0.1 M IPTG was used to induce FEN1 expression and the temperature lowered to 25°C. After an overnight incubation, the cells are harvested by centrifugation at 4000xg, for twenty minutes at 10°C. Cells are stored in 10 g pellets at -80°C.

Cell Lysis

For Biochemical assays, frozen cell paste was resuspended in lysis buffer (5 ml per gram of paste). To break down the bacterial cell wall Lysozyme (200 μg/ml) was added and left to incubate for twenty minutes. Protease activity is inhibited by the addition of Phenylmethanesulphonylfluoride (PMSF) (23 μg/ml) and Sodium deoxycholate (500 μg/ml) is added to break down the cell membrane. The solution was left stirring for twenty minutes before a final addition of DTT (1 mM) to break disulphide bonds. Sonication was used to reduce cell viscosity and disrupt the cell membrane. Cells were sonicated on maximum power for twenty seconds. This was repeated 2-3 times depending on how viscous the original solution was. Cells debris was then pelleted by centrifugation at 75600xg. Any remaining nuclease – DNA interactions were disrupted by adding 0.5 M Ammonium Sulphate. 5% w/v polyethyleneimine (PEI) was added and left stirring for 15 minutes to remove nucleic acids. Centrifugation at 75600xg for fifteen minutes pelleted the PEI and nucleic acids which was then discarded.

For crystallography frozen cell paste was resuspended in buffer (50 mM Tris pH 8.0, 0.2 M NaCl) and the cells disrupted by sonication at maximum power for twenty seconds. The sample is sonicated 2-3 times depending on viscosity. Cell debris was removed by centrifugation in at 70000xg for 15 minutes at 4°C. Supernatant is collected and the concentration checked. The cell pellet is discarded.

Ion Exchange and Affinity Chromatography

For biochemical assays, the protein was dialysed overnight into phosphate buffer with 50 mM NaCl. The sample were loaded onto the columns in the same buffer. Chromatography was performed using a peristaltic pump. First a cation exchanger (Biorad S or Hi TRAP GE Healthcare SP) was used followed by an affinity chromatography column (Heparin-GE Healthcare). Fractions were eluted over a 0.05-0.1 M NaCl gradient in 20x the volume of the column itself (5 ml). Fractions were analysed using SDS-PAGE. Any remaining contaminants were removed using size exclusion chromatography using a Superdex 200 16/600. Protein samples were concentrates to a volume of 3-5 ml and buffer exchanged into 50 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT, 5% Glycerol. 2ml fractions were collected and analysed using UV and SDS-PAGE.

For crystallography the protein was loaded straight onto a Heparin HP affinity column in a 50 mM Tris pH 8.0, 0.2 M NaCl buffer. Chromatography was performed on the AKTA purifying system. The fractions were eluted over a 0 – 0.1 M NaCl gradient in 15x the column volume itself (5 ml). 2.5 ml fractions were collected and protein elution was evaluated using the fixed wavelength (280 nm) UV monitor (U9-L). A clear peak was seen on the UV readout allowing specific fractions to be analysed for protein using Bio-Rad assay. Fractions containing protein were pooled and concentrated using a Vivaspin 30000. The concentrated sample was then diluted into 30 ml 50 mM MES pH 6.3 and loaded onto a Resource S Cation Exchanger. Chromatography was performed on the AKTA purifying system and fractions were eluted over a 0.05 – 0.25 M NaCl gradient in 10x the volume of the column itself (6 ml). 2.5 ml fractions were collected and from the UV readout specific fractions were analysed for protein using Bio-Rad assay. Fractions containing protein were pooled and concentrated using a Vivaspin 500.

Protein Storage

For biochemical assay concentrated protein was stored in 50% glycerol in the -20°C freezer or snap frozen in liquid nitrogen for storage at -80°C.

When protein was to be used in crystal trials it was taken straight from purification and buffer exchanged for use the same day. If it was not possible to go straight to crystal trials the protein was kept at 4°C overnight for use the next day.

Mammalian Cell Culture

The effects of overexpression of FEN1 and FEN1 mutation were investigated in the A549 lung adenocarcinoma cell line. Cells were provided by a Thomas Jones a member of the Bryant lab group. All cell work is carried out in a tissue culture hood.

Passaging Cells

Media (DMEM, FCS (50 ml), non-essential amino acids (5.5 ml)) and trypsin are always warmed in a water bath for a minimum of thirty minutes prior to use.

Current media was discarded into precept. The cells were washed twice in PBS (10 ml) to completely remove any remaining media. Pre-warmed trypsin (1 ml) was added to the flask and swirled gently to ensure full coverage. The flask was then incubated with the trypsin at 37°C for 5 minutes to allow complete removal of cells from the surface. Once all the cells were suspended, media (9 ml) was added to the flask and mixed thoroughly. A new flask was labelled with name, date, cell line and passage number before the addition of 1.3 ml of cells (1 in 6 split). Media (8.7 ml) was then added to the flask to give a total volume of 10 ml. Cells were split twice a week.

Counting Cells

To plate many cells specifically we need to be able to reliably count cells using a haemocytometer coverslip. 7 μl of trypsinised cells were added to the edge of a haemocytometer coverslip mounted on the haemocytometer chamber. The haemocytometer chamber contains four quadrants. The cells in each quadrant are counted and the average of the four quadrants used to calculate the number of cells per ml. The grid of the haemocytometer chamber is 1mm x 1mm in area and 0.1 mm deep i.e. 10 -4 ml. Therefore, if the average count over the four grids was 60 then there would be 60 x 104 cells per ml.

Transfection of Mammalian Cells

A549 cell line was transfected with WT and D179K FEN1 in pDEST 12.2 plasmid. The transfection reagent used was FuGENE HD and the protocol for transfection was taken from the promega website <http://www.promega.com/techserv/tools/FugeneHdTool/> This protocol prepares sufficient amounts of DNA/FuGENE HD reagent to transfect 12 wells at 3 ml/well.

The day before transfection 3 x 105 cells were plated in each well of two 6 well plates in 3 ml of media (DMEM, FCS (50 ml), non-essential amino acids (5.5 ml)).

155 μl of each required concentration of DNA (0 μg/μl, 0.5 μg/μl, 1 μg/μl, 3 μg/μl, 5 μg/μl) was prepared and 9.9 μl of FuGENE® HD reagent added to each individual concentration of DNA. The reagents were mixed carefully by pipetting or by vortexing briefly and then incubated at room temperature for 5 minutes before the addition of 150 μl of the complex per well to the cells and mix thoroughly.

After 24 hours if the cells are confluent in the untransfected sample, trypsinise and transfer to 90 mm petri dishes to allow a 48-hour transfection period.

Harvesting Cells

Cells in 90 mm dishes were trypsinised and diluted to 10 ml using media. Cells were spun at 3000xg for 3 minutes to allow pellet formation. Cell pellets were washed in PBS (~5 ml) and centrifuged again. The cell pellet was then resuspended in 1 ml PBS and transferred to an Eppendorf. Samples were spun at 2000xRPM, for 10 minutes at 4°C, before being stored at -80°C.

Cell Lysis / Protein Extraction

Frozen stocks were thawed on ice and resuspended in 50 μl lysis buffer (1% v/v PMSF, 1% v/v Protease Inhibitors, 1% v/v Phosphatase Inhibitors, 1 x RIPA Buffer, made to volume with ddH2O). Once resuspended fully the samples are left to incubate on ice for 30 minutes with vortexing every 10 minutes. Due to FEN1 being a DNA binding protein the samples were then passed through a 25G needle. Cell debris was then pelleted via centrifugation at 13,000 RPM for 10 minutes at 4°C. The supernatant was then transferred to a sterile Eppendorf.

Total protein concentration in the cell extract was measured against a BSA standard curve of absorbance at 595 nm. This quantification allowed equal loading of the samples on to an SDS Page gel. The gel was then transferred to a nitrocellulose membrane for western blotting with an anti FEN1 antibody.

Crystallography

Preparation of Protein and 5ov4.

For crystallography protein was prepared as described above to a concentration of 9 mg/ml in 5 mM Tris, 50 mM NaCl.

5ov4 was prepared in annealing buffer (10 mM MES pH6.5 and 50 mM KCl) to a final concentration of 2.2 mM. In order to anneal the DNA strands the oligo was heated to 95°C for 10 minutes, with brief centrifugation ever two minutes, before cooling to room temperature. FEN1 protein was mixed with 5ov4 in a 1:1 molar ratio. In most of the initial screens and in all optimisations 100 mM MgCl2 and 100 mM KCl was also added to the protein:DNA solution.

Setting up screens

Initial crystal trials were produced by the Hydra II Robot in 96 well plates. Sitting drop screens were used with the large drop containing 20 μl of screening conditions and the small drop containing 200 nl of protein mixed with 200 nl screening condition. Crystallisation screens used included Morpheus, PACT and JCSG.

For optimisation of crystals the hanging drop method was used. In which a 24 well plate was used with 1 ml of screening condition in each well. 1 μl of protein was mixed with 1 μl of the well condition on a siliconized coverslip which was then suspended and sealed above the well using oil. Optimisation of conditions typically utilised a varying of pH and precipitation concentration (PEG, Ammonium Sulphate).

**Results and Discussion**

The first aim of my project was to crystallise a FEN1- substrate complex. To achieve this, we needed a FEN1 mutant that would allow the binding of a substrate but was catalytically inactive, preventing substrate cleavage to product. A D153K mutation in bacteriophage T5 flap endonuclease was found to allow the binding but not cleavage of the DNA substrate 5ov4 and this T5 mutant residue maps to the residue D179 in humans (AlMalki et al., 2016). Work in the Sayers lab showed that the D179K mutation in humans gave the same catalytic properties as seen in the T5 enzyme (Oates, 2016) and for this reason I began growth and purification of the D179K mutant with the aim of getting a crystal structure. In addition of the requirement of catalytic inactivity, it is also essential that the protein can crystallise. Previous literature and work in the University of Sheffield Crystallography department has shown that truncation of the C-terminal PCNA binding domain favours crystal grown as the PCNA binding domain is thought to be disordered and disordered regions do not form a fixed structure for crystallisation. For this reason, I have used a 38 kDa truncated WT and D179K FEN1 for crystal trials.

Optimisation of growth conditions.

The first optimisations varied incubation temperature in order to maximise FEN1 expression in *E.coli*. There is a direct correlation between *E.coli* growth and temperature, with optimal growth occurring at body temperature (37°C). For this reason *E.coli* is often incubated at 37°C, giving optimal bacterial growth and in theory producing the highest possible expression levels of FEN1. However, higher temperatures do not always favour protein solubility. Lower temperatures allow the protein more time to fold increasing protein solubility. In addition, lower temperatures have been shown to decrease the amount of protein accumulating in inclusion bodies.

Three conditions were tested on a small scale (50 ml). Firstly, incubation at 37°C both pre and post IPTG induction. Secondly, incubation at 37°C pre ITPG induction followed by incubation at 25°C. The third and final condition was incubation at 25°C both pre and post IPTG induction. Samples were taken at regular intervals over a 48-hour period and run on an SDS-PAGE gel for analysis. Figure 19 shows that overexpression of FEN1 can be achieved and that incubation at 37°C pre ITPG induction followed by incubation at 25°C marginally gave best expression levels.

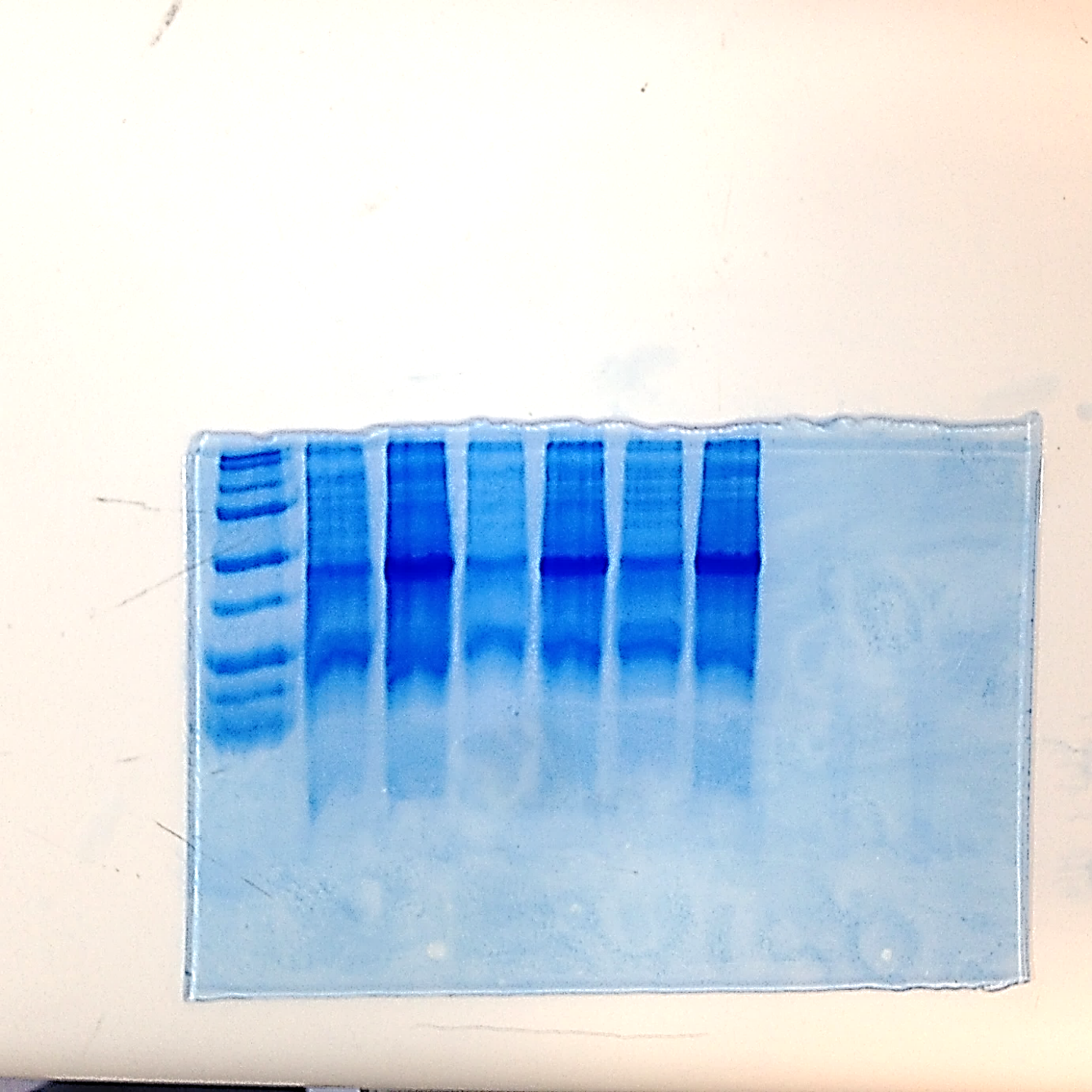
The second optimisation varied the glucose concentration used in the growth media to maximise FEN1 expression levels. Glucose is the preferred carbon source of *E.coli.* Whilst glucose is present no other sugars will be used, this includes lactose and IPTG. As a result, glucose can be used to repress the induction of the lac promoter by lactose which is often found in rich media such as 5YT. This of course needs to be a balance as too much glucose will affect the induction of protein growth by IPTG. Three conditions were tested 0.05% glucose, 0.1% glucose and 0.25% glucose. Samples were collected over a 48-hour period and run on an SDS-PAGE shown in figure 20. As you can see sample 3 (0.25% glucose) inhibited growth up to 24 hours where as 0.05% and 0.1% glucose were showing FEN1 expression after 7 hours. There was no noticeable difference between FEN1 expression at 0.05% and 0.1% glucose, most likely due to the very small difference in concentration however, for future growth we decided to use 0.1% glucose as a standard.

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| Figure 19: Optimisation of Temperature for WT truncated FEN1 expression. Sample 1 - 37°C incubation both pre and post induction. Sample 2 - 37°C incubation pre induction and 25°C incubation post induction. Sample 3 - 25°C incubation both pre and post induction. |
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| Figure 20: Optimisation of Glucose Concentration for WT truncated FEN1 expression. Sample 1 – 0.05% glucose. Sample 2 – 0.1% glucose. Sample 3 – 0.25% glucose. |

Overexpression of D179K and WT FEN1

D179K and WT FEN1 in Pet21a+ were transformed and overexpressed in 5YT as described in the materials and methods section. To check overexpression had been achieved samples were taken over a 24-hour period and analysed using SDS-PAGE (figure 21). There is a very noticeable difference in the overexpression of the WT protein over the mutant D179K protein. The WT samples show clear overexpression whereas the D179K samples seem to have no change in concentration from 3 hours to 24 hours. I believe the reason for this may be that it is not in the best interest of *E.coli* for it to overexpress the protein. Although we have manipulated the bacteria to do so, it was discussed in detail in the introduction that FEN1 is essential for DNA replication and repair. Disruptions to the enzymatic activities of FEN1 cause disastrous consequences for genome integrity. Therefore it is unsurprising that *E.coli* may prevent overexpression of a FEN1 mutant that has no catalytic activity and if allowed to overexpress may outcompete WT FEN1 giving DNA damage.

Despite not achieving the overexpression desired, the cell pellets obtained from all flasks were purified and D179K FEN1 protein was obtained at adequate concentrations.



Protein Marker

Flask 1 – 3 hours

Flask 2 – 3 hours

Flask 3 – 3 hours

Flask 1 – 24 hours

Flask 2 – 24 hours

Flask 3 – 24 hours

50

37

|  |
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| A  B  100  75 |
| Figure 21: Overexpression of WT and D179K truncated FEN1. Three 1 litre flasks were grown, and samples were taken up to 24 hours post induction. Biorad Precision Plus protein marker was used. Gel A is representative of WT FEN1 overexpression and gel B is representative of D179K FEN1 overexpression. |

Purification of D179K FEN1

For crystallography, truncated D179K FEN1 was purified as outlined in the methodology section. Fractions from the Heparin and Resource S columns were collected on the AKTA and analysed using UV spectroscopy. The UV spectroscopy readout for both the Heparin and Resource S columns are shown in figures 22 and 23. From these graphs, protein elution is represented as a peak in the absorbance at 280nm, shown by the dark blue line on the graphs. Conductivity is shown in brown and confirms the salt gradient formation for elution. In both instances fractions were selected based on the peaks shown and then tested for protein using a Bradford assay of absorption at 595 nm (figure 22 and 23).

Throughout the purification process samples were taken to ensure that the protein we were seeing was in fact FEN1. At the end of the purification these samples were run on an SDS-PAGE gel which is shown in figure 24. The gel shows a band around 38 kDa which is representative of truncated FEN1. There are contaminants in the purified product shown in lane 7. Although proteins used for crystallography should be as pure as possible as impurities may prevent or inhibit crystal growth (Spencer and Nowick, 2015) in general a protrin that is over 90% pure is seen as sufficient for the commencement of crystallization screens (Rowlett, 2005). If this protein was required for biochemical assays or there were issues with crystallisation believed to be because of the impurities present, then gel filtration chromatography could be used. Separation of the partially purified product from figure 24 was by molecular mass, would exclude many of the contaminants leaving a purified product. The use of gel filtration chromatography would also highlight if the protein dimerises or aggregates as the protein-protein interactions formed would alter the molecular weight of the product.

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| |  |  | | --- | --- | | Fraction | Abs: 595 nm | | 20 | 0.26 | | 21 | 0.43 | | 22 | 0.60 | | 23 | 0.53 | | 24 | 0.48 | | 25 | 0.40 | | 26 | 0.40 | |
| **Figure 22: UV Spectroscopy readout from the Heparin column**.  The protein was purified using a gradient elution from 0-1 M NaCl over 15 x the column volume (5 ml). The initial peak at Abs 280 nm over fractions 1 – 10 corresponds to the naturally binding proteins in the lysate, where as the second peak of absorbance over fractions 20-26 represents the elution of FEN1 which has a higher affinity for the column. The conductivity is shown in brown and confirms the salt gradient formation. The % gradient of buffer B (50 mM Tris pH 8.0, 1 mM NaCl) is shown in green. The protein from fractions 20 – 26 was tested for protein using absorbance at 595 nm, the results of which are shown in the table above, with all fractions containing protein. |

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| |  |  | | --- | --- | | Fraction | Abs: 595nm | | 12 | 0.00 | | 13 | 0.54 | | 14 | 0.31 | | 15 | 0.01 | |
| **Figure 23: UV Spectroscopy readout from Resource S column**.  The protein was purified using a gradient elution from 0-1 M NaCl over 15 x the column volume (5 ml). The peak of absorbance over fractions 13 - 15 represents the elution of FEN1. The conductivity is shown in brown and confirms the salt gradient formation. The % gradient of buffer B (50 mM MES pH 6.8, 1 mM NaCl) is shown in green. The protein from fractions 12 - 15 was tested for protein using absorbance at 595 nm, the results of which are shown in the table above, with fractions 13 – 15 containing protein. |

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| Figure 24: SDS-PAGE of purification samples.  Total cell – resuspended cell pellet. Total soluble – soluble fraction post centrifugation. Post Heparin – protein sample eluted from the heparin column. Precipitation pellet – during concentration after the heparin column, there was some precipitation. The sample was spun down and a small pellet formed, this was resuspended and run on the gel. Pre-Resource S – the protein sample loaded on to the resource s column. Purified Product – The final protein sample, concentrated and buffer exchanged ready for crystallisation. Mark 12 Protein marker was used, and the band believed to be representative of FEN1 is highlighted. |

Crystal Screens

The purified product from figure 24 was mixed in a 1:1 molar ratio with 5ov4 DNA. Three crystal screens were set up, one with FEN1 and 5ov4 only, another with FEN1, 5ov4 and 100 mM KCl and finally a third screen with FEN1, 5ov4, 100 mM KCl and 100 mM MgCl2. The potassium was added as this has been shown to increase DNA binding in FEN1. As I highlighted in the introduction magnesium is essential to FEN1 activity and is therefore added to ensure that FEN1 is in an active confirmation and allow us to see the threaded DNA.

Each of the three conditions were added to three 96 well plates (PACT, JCSG and Morpheus) using the Hydra robot II. The plates were left at room temperature to grow and crystal formation was monitored over the following weeks. Interestingly the crystal growth was dependent on the presence of the magnesium. Screens that only contained FEN1 and 5ov4 showed little to no crystal growth with the formation of some microcrystals over time. The plates that had added potassium showed better microcrystal growth, however there were no defined crystals. On the contrary the plates containing magnesium and potassium showed clear crystal growth in the wells that had microcrystals in the potassium only screens.

The best crystals we saw were in well A5 of the PACT screen which uses Poly Ethene Glycol as the precipitate and varies the pH to find suitable crystallisation conditions. The favourable conditions in well A5 were 25% w/v PEG 1500 and 0.1 M SPG pH 8.0 (2:7:7 molar ratio of succinic acid, sodium hydroxide phosphate and glycine) containing FEN1, 5ov4, 100 mM KCl and 100 mM MgCl2. Figure 25 shows a photo captures form this well and the colourful polarisation of the crystals usually confirms the presence of protein and not salt crystals.

Other crystals were grown in the Morpheus screen which uses a combination of 49 low molecular weight ligands and a range of pH, PEGs and salt additives. Although these crystals were not as defined and would require further optimisation before they could be picked and sent to diamond for analysis. As can be seen in figure 25 there were favourable conditions in row C of the Morpheus screen that allowed crystal formation. The conditions for this row are show in table 4. Every condition contains the ligand NPS, which is a mix of sodium nitrate, disodium hydrogen phosphate and ammonium sulfate. It is hoped that optimisation of these crystals over varying pH ranges and varying amounts of precipitant will allow better crystal formation.

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| Figure 25: Crystal growth in 96 well crystal screens.  All wells shown contained FEN1, 5ov4, 100 mM KCl and 100 mM MgCl2 A - Well A5 of the PACT screen: conditions in this screen were 25% w/v PEG 1500 and 0.1 M SPG pH 8.0 (2:7:7 molar ratio of succinic acid, sodium hydroxide phosphate and glycine). B – Well C4 of Morpheus (0.09 M NPS, 0.1 M Imidazole; MES monohydrate (acid) pH 6.5, 37.5% v/v 25% v/v MPD; 25% PEG 1000; 25% w/v PEG 3350). C – Well C5 of the Morpheus screen (0.09 M NPS, Sodium HEPES; MOPS (acid) pH 7.5, 30% v/v 40% v/v PEG 500\* MME; 20 % w/v PEG 20000) D – Well C7 of the Morpheus screen (0.09 M NPS, Sodium HEPES; MOPS (acid) pH 7.5, 30% v/v 40% v/v Glycerol; 20% w/v PEG 4000). E – Well C9 of the Morpheus screen (0.09 M NPS, Tris (base); BICINE pH 8.5,30% v/v 40% v/v PEG 500\* MME; 20 % w/v PEG 20000) F – Well C10 of the Morpheus screen (0.09 M NPS, Tris (base); BICINE pH 8.5, 30% v/v 40% v/v Ethylene glycol; 20 % w/v PEG 8000) |

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| Table 4: The Crystallisation Conditions in Row C of the Morpheus HT-96 Screen |
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| Table 5: Optimisation conditions |
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Crystal Optimisation

Optimisation of the crystals in well A5 of the PACT plate (figure 25A) was achieved using the hanging drop method as described in the materials and methods section. The optimisation conditions used are shown in table 5. In this optimisation screen, I varied the amount of precipitant PEG 1500 and the pH of the SPG buffer. Although the crystals I the original screen were of a considerable size and had good morphology it is always good practice to get crystals over differing pH ranges as they tend to give varying levels of diffraction.

Figure 26 shows a sample of some of the crystals grown from the optimisation screens. I successfully grew crystals at pH 7.5, 8 and 8.5, over a PEG 1500 concentration range of 22.5% - 32.5% w/v. These crystals were in 1μl drops in comparison to the 1nl drops used in the sitting drop screens and therefore as expected the crystals are much bigger. In figure 26B and 26E you can see that there are clusters of crystals rather than individual crystals. Although this is not ideal the crystals can be separated by stabbing them with an acupuncture needle allowing them to be analysed.

Diffraction Data

Ideally the crystals from the original screen (figure 25) would have been picked and sent for analysis before optimisation, however with a large gap between Diamond trips this was not possible. As a result, two crystals from well A5 of the PACT screen (Figure 25A) and two crystals from well C2 of the optimisation plate (Figure 26C) were picked at the same time. Ordinarily the crystals would be cryogenically protected using a cryogenic solution, however I was not able to make the required cryogenic solution (0.1 M SPG, % w/v PEG 1500, 30% Ethylene Glycol, 100 mM KCl, 100 mM MgCl2) as it kept precipitating so instead the crystals were soaked in oil before being stored in liquid nitrogen.

Diffraction data was collected from all four crystals and unfortunately the large spacing of the diffraction data suggested that the crystals were in fact salt (figure 27). There are however a few things to note when considering this result. Firstly, it was noticeable that the crystals once mounted to the laser had a fluffy appearance that I can only assume is caused by ice and this may have affected the diffraction data. This will have almost certainly have been a result of the lack of cryogenic protectant, which may cause issues in the future.

In addition, why was it that the crystals grew preferentially in the presence on magnesium? Originally, I had believed this was because magnesium coordinates the acidic residues in the active site, which when not bound to metal give the active site an overall net negative charge. This negative charge will repel the negative phosphate backbone of DNA. However, considering these results I believe the sodium phosphate in the SPG buffer was reacting with the magnesium and driving the formation of magnesium phosphate crystals. This would also explain the polarisation of the crystals, which again lead us to believe that the crystals are protein however phosphate crystals are also brightly coloured under polarised light.

Although disappointing this has given other leads that I can follow, for example the optimisation of the C-row crystals in the original Morpheus screen (figure 25).

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| Figure 26: Crystals obtained from the optimisation screen.  All wells shown contained FEN1, 5ov4, 100 mM KCl and 100 mM MgCl2. A – Conditions: 0.1 M SPG pH 7.5, 22.5% PEG 1500. B – Conditions: 0.1 M SPG pH 7.5, 30% PEG 1500. C – Conditions: 0.1 M SPG pH 8.0, 22.5% PEG 1500. D – Conditions: 0.1 M SPG pH 8.0, 27.5% PEG 1500. E: Conditions – 0.1 M SPG pH 8.0, 32.5% PEG 1500. F: Conditions – 0.1 M SPG pH 8.5, 30% PEG 1500. |

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| Figure 27: Diffraction Data.  The diffraction data obtained from the analysis of a crystal picked from well A5 of the PACT screen (Figure 25A). Protein crystals exhibit many closely spaced diffraction spots, while for salt, with its small unit cell, the reflections are far apart (and much stronger) The image above shows wide spaced diffraction spots, indicating the crystal was salt. |

Other Active Site Mutants

The mutation of aspartic acid to lysine at residue 179 gives a catalytically inactive form of FEN1. Residue 179 maps to the active site of FEN1, where it is one of 7 conserved acidic residues (figure 6). To see if mutation of the remaining 6 would also give changes to catalytic ability, I designed primers for PCR mutagenesis of WT FEN1 (Table 6). These primers will allow the mutagenesis of the large bulky aspartic acid and glutamic acid residues to a smaller lysine residue. This will not only give structural differences in the active site but will also induce a charge swap to a positively charged amino acid, which will likely alter the active site binding with the negatively charged phosphate backbone of DNA. Once these mutants have been made and their DNA sequences confirmed they can be overexpressed in Ecoli for use in biochemical assays assessing their enzymatic activity. This will further our knowledge of the importance of these active site amino acids for enzymatic activity and potentially create another mutant that is also able to bind but not cleave DNA for use in further crystal trial.

Table 6: Primer sequence for PCR mutagenesis. Bases highlighted in red represent the amino acid substitutions used to incorporate the mutation.

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| **Mutation** | **Primer Sequence** |
| D233K | TTCTGCTGGGTAGCAAATATTGTGAAAGCATT  AATGCTTTCACAATATTTGCTACCCAGCAGAA |
| D179K | TGCAGCAGCCACCGAAAAAATGGATTGTCTGACCTTT  CCAAAGGTCAGACAATCCATATCTTCGGTGGCTGCTGCA |
| E160K | TGCACCGAGCGAAGCCAAAGCAAGCTGTGCAGCACTGGTT  AACCAGTGCTGCACAGCTTGCTTTGGCTTCGCTCGGTGCA |
| E158K | ATCTGGATGCACCGAGCAAAGCCGAAGCAAGCTGTGCAGCACT  AGTGCTGCACAGCTTGCTTCGGCTTTGCTCGGTGCATCCAGAT |
| D86K | AAACCGGTGTATGTTTTTAAAGGTAAACCTCCGCAGCTGAA  TTCAGCTGCGGAGGTTTACCTTTAAAAACATACACCGGTTT |

FEN1 Overexpression in Human lung cancer A549 Cell Line

A549 cells are adenocarcinomic human lung cancer cells Derived from a 58-year-old Caucasian male in 1972. These cells are adherent and form a monolayer as well as being efficient for transfection, making them suitable for FEN1 overexpression. As mentioned in my introduction, lung cancer is the most prevalent cause of cancer related death worldwide and 85% of these lung cancer cases are categorized as non-small cell lung cancer (NSCLC). Adenocarcinomas account for 50% of NSCLC cases and for this reason this cell line is a good representation of lung cancer incidences and an area that requires further research.

Overexpression of FEN1

It is well documented that FEN1 is overexpressed in a variety of cancer types and for this reason is now seen as an anticancer target. Inhibitors that will bind FEN1 and as such reduce FEN1 levels within the cell may provide a potential therapeutic benefit. Studying reduced FEN1 levels in human cells however is not possible using siRNA as FEN1 is essential for cells and therefore without it they will die. Instead of studying FEN1 knockouts we decided to use overexpression of the D179K FEN1 mutant from the crystal trials which lacks nuclease activity. When transfected into A549 cells we hope to see a change in phenotype in comparison to cells with overexpressed wild type FEN1.

Cells were transfected with either WT or D179K FEN1 (0 μg – 5 μg) using the FuGENE HD transfection reagent as detailed in the materials and methods. For both the WT and mutant DNA cells were kept in 6 well plates for 24 hours and then transferred to 90mm petri dishes for a further 24 hours. Cells were harvested and lysed to give a total cell extract. The representative levels of FEN1 were analysed using western blot detection with ORC2 as a loading control. The antibody used to detect FEN1 for this western blot was too concentrated making it very hard to analyse the results. However, further optimisation of the antibody concentration proved a 1:2500 dilution was optimal and as such the experiment can now be repeated and re-analysed.

Interestingly over the 48-hour period there was no obvious difference in phenotype between the cells overexpressing WT or D179K FEN1. There are of course limitations with this method of overexpression as we rely on competitive binding of the transfected FEN1 over the endogenous FEN1. It is not possible to tell if the cells are indeed using the transfected DNA or if they are still using the WT endogenous FEN1.

The data produced in this project has shown that A549 cells are able to survive up to 48 hours when overexpressing D179K FEN1. Future work will be aimed at looking at any DNA damage that has occurred in these cells due to overexpression of D179K FEN1. As mentioned merely transfecting cells and hoping that the transfected DNA is preferentially used is not fully reliable so for future assays developing CRISPR interference assays will more accurately control overexpression of FEN1.

Conclusions

FEN1 plays a vital role in DNA repair and replication and as such is essential for genome stability. Its overexpression across a variety of cancer types has led to it being a very desirable drug target both as a monotherapy and in combination with chemotherapy where it is thought it could enhance the effects of DNA damage in cancer cells.

During this project we successfully overexpressed truncated WT and D179K FEN1 and purified the product to a suitable level for crystallisation. Although crystals were formed there will still impurities in the product and this could have been improved with the use of gel filtration chromatography or the addition of a protein tag. The latter will also quicken the purification process as affinity purification could be used. This is beneficial to the purification of FEN1 as we discovered it to be unstable once purified, especially at high concentrations.

It is clear from this thesis that FEN1 crystals can be formed, however, further optimisation and careful consideration is required with regards to optimisation conditions to encourage crystal formation. One of the difficulties experienced during this project was the cryoprotection of the crystals prior to them being frozen and sent for analysis. This issue may have been due to the specific conditions in the crystallisation solution required, however, it was clear that the use of oil alone is not sufficient as the crystals appeared fluffy in appearance when mounted on the synchrotron, most likely due to ice formation.

In addition, other oligonucleotides should be screened as DNA substrates. Differing the lengths of the DNA substrates will provide crystal structures of FEN1 bound in different conformations providing further structural information. With a greater library of crystal structures, there will be more targets in place to start drug design.

The primers designed and shown in this thesis should be used in PCR mutagenesis to create a library of active site mutants. These mutants can be used in assays to assess the importance of active site amino acids and with a library of other mutants that may also lack nuclease activity, they too can be used in crystallisation studies, giving a potential for further drug targets.

We do not yet know if it is possible to overexpress FEN1 in human cells, but it was concluded from the transfection we did that no obvious phenotype is seen between the transfected WT or D179K FEN1 cells when using the FUgene transfection reagent. Further transfections and monitoring of the cells for longer periods using immunofluorescence and western blotting will help to improve our understanding.

My research has shown that there are many questions still unanswered with regards to FEN1. Although much of what we know has come from studies of FEN1 homologues in bacteria, fungi and viruses, it is important to replicate these findings in human FEN1 models and as such expand our understanding of its role in DNA replication and repair, its overexpression in cancer cells and the implications this has for therapeutic reagents such as chemotherapy.

Further Work

The continuing aim of this research is to develop a crystal structure of FEN1 in combination with a few different DNA substrates. Jason Wilson will be continuing with the crystallographic studies I have done, laying down further screens and optimising the crystals I found in the Morpheus screens. The focus will remain on using the catalytically inactive truncated D179K FEN1 mutant to obtain a crystal structure of a FEN1: substrate complex. This complex will be of interest for further use in structure-based drug design and small molecule screens. If we can find a suitable inhibitor that will bind FEN1 and its substrate, it is possible for it to become trapped in this complex rendering it inactive. Furthermore, a molecule that binds the complex will be much more likely to show high levels of specificity producing a drug which will be more viable for use in clinical trials. This is very important as previous attempts at developing a FEN1 inhibitor have all been unsuccessful. There are no drugs currently in clinical trial despite there being a lot of research done to find an inhibitor in other labs and by Astra Zenica. The reason for the lack of success is because many of the drugs developed were found to be nonspecific, binding to other DNA binding proteins and causing numerous side effects.

To make the purification of FEN1 simpler and quicker and ensure fewer impurities it would be worth considering a tagged version of FEN1 that can be cleaved post purification. The Pet21-a+ vector has a T7 tag on the N-terminus, however this wasn’t utilised in the plasmids provided for this project. The use of a tag would not only make the purification simpler but hold the potential to increase yield and solubility which is a reoccurring issue with the purification of FEN1.

In addition, there is a need to develop a more accurate way to monitor FEN1 overexpression. This would not only allow us to fully analyse DNA damage due to FEN1 mutation but will also provide an in vivo model in which to screen potential drug candidates. Whilst original work will be done in vitro, this can then be transferred in vivo to see if any of these molecules give an apparent phenotype in a variety of cancer cells. The WT and D179K plasmids from this project can further be used to analyse the importance of FEN1 in cancer cells, with longer transfection periods and monitoring of any phenotypic differences or DNA damage. With the development of other active site mutants, they too can be transfected into the cell, again assessing the importance of the active site amino acids to the nuclease. Furthermore, if cancer cell lines can be transfected with D179K, it would then be of interest to treat these cells with known chemotherapeutic agents. If the cancer cell lines become sensitised to the chemotherapeutic agents transfected with the D179K FEN1, it would further enforce the need to invest in FEN1 inhibitors for use in combination therapy.

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