Generation of a novel endogenously StrepII/FLAG tagged system for the identification of the vertebrate FANCJ associated proteins.

A thesis submitted at the University of Oxford for the degree of Master of Science (by Research)

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ABSTRACT

Fanconi Anaemia (FA) is a rare inherited chromosomal instability disorder characterized by developmental abnormalities, bone marrow failure, increased risk of developing cancer and an increased cellular sensitivity to DNA interstrand crosslinking compounds, including the anticancer drugs, cisplatin, nitrogen mustard and mitomycin C. From a therapeutic point of view, it is important to understand how cells respond to and repair DNA damage caused by crosslinking compounds, used to treat cancer. To date, fifteen different proteins involved in a common pathway, known as the FA pathway have been identified and are known to respond to, and influence repair of interstrand crosslinks (ICLs). So far, the role of these proteins in ICL repair remains elusive. To gain insights into the molecular basis of ICL repair, we chose to study the FA-associated helicase FANCJ, as it is known to bind and metabolize a variety of DNA substrates, implicating it in maintenance of genomic stability. Here, I report a novel genetic-proteomic approach to study FANCJ. This system uses epitope tagged FANCJ expressed from its chromosomal locus, in chicken DT40 cells, kept under the control of its endogenous promoter. Tandem affinity purification is employed to purify and isolate the tagged protein and identify interacting partners of interest. To date, I have generated, characterized and validated epitope tagged FANCJ cell lines. Pilot immunoprecipitations were carried out to establish the efficiency and reproducibility of the immunoprecipitations (IPs). Analysis by mass spectrometry revealed the presence of epitope tagged FANCJ in scaled-up immunoprecipitations. In the future, these cell lines will be used to identify and characterize the FANCJ interactome.

CONTENTS:

ABBREVIATIONS

AML	Acute Myeloid Leukemia
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine Triphosphate
ATR	ATM and Rad3 related
BRCA	Breast Cancer associated protein
BSR	Blasticidin resistance
CP	Cisplatin
DNA	Deoxyribose Nucleic Acid
FA	Fanconi Anaemia
FAN1	Fanconi Associated Nuclease 1
FANC	Fanconi Anaemia Complementation Group
H2AX	Histone family member 2 variant
HR	Homologous Recombination
HRR	Homologous Recombinational Repair
ICL	Interstrand Crosslink
IP	Protein Immunoprecipitation
MHF	Major Histone fold heterodimer
MLH	MutL Homolog
MMC	Mitomycin C
MMR	Mismatch Repair
MS	Mass Spectrometry
NEO	Neomycin
NER	Nucleotide Excision Repair
PTM	Post Translational Modification
RPA	Replication Protein A
SF Tag	StrepII FLAG Tag
SF-TAP	StrepII FLAG Tandem Affinity Purification
SDS-PAGE	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis
TLS	Translesion DNA Synthesis

Chapter 1: INTRODUCTION

Fanconi anaemia (FA) is an autosomal recessive and X-linked hereditary disorder characterized by congenital abnormalities, progressive bone marrow failure and increased cancer susceptibility. Cells from FA patients exhibit elevated levels of chromosomal aberrations, including characteristic abnormalities known as radial chromosomes (Sasaki and Tonomura 1973). A hallmark feature of FA cells and patients is their hypersensitivity to DNA interstrand crosslinking agents, such as mitomycin C and cisplatin. Upon treatment with crosslinking agents, FA cells display increased genomic aberrations such as chromosomal breaks and radial chromosomes. This suggests that defective interstrand crosslink (ICL) repair might at least in part underlie the clinical and cellular phenotypes associated with FA, and implicates the protein products of the FA genes in ICL repair.

The primary diagnostic method for FA is the chromosomal aberration test, which comprises the use of crosslinking agents as mutagens, which serve as indicators for the diagnosis of FA. Other diagnostic methods include cell-cycle analysis on the patient lymphocytes exposed to crosslinking agents in order to discriminate between FA and non-FA individuals. This method is based on the fact that the duration of the G2/M phase is increased in FA cells as compared to unaffected cells (Seyschab et al 1995). Also, if the specific complementation group and/or mutations are known within the family, testing for the presence of the mutation can be used for diagnosis (Auerbach 2009).

Initial identification of the proteins involved in FA was carried out using cell fusion or cell complementation experiments. Here, FA cell lines derived from different patients were used to complement each other's crosslinker sensitivity. This technique was used to define most FA complementation groups, e.g. FANCA, FANCB, FANCC, FANCD, FANCE, FANCF, FANCG, FANCI and FANCJ (Strathdee et al 1992a) (Joenje et al 1995, Joenje et al 1997, Levitus et al 2004). Later, cloning strategies were used for the identification of the gene defects involved in the various FA complementation groups (Strathdee et al 1992b, Lo Ten Foe et al 1996, de Winter et al 1998, de Winter et al 2000a, de Winter et al 2000b).

So far, 15 FA genes have been identified. Biallelic mutations in any one of 14 genes are responsible for FA. Of the 14 genes, FANCB is located on the X-chromosome. Female carriers have no phenotype but males who inherit the mutated copy of X-chromosome are affected. The 15th gene (FANCP) is mutated in an FA-like syndrome (Vaz et al 2010). The protein products of FA genes (Table 1) interact at sites of DNA damage during S-phase to help overcome replication stress and maintain genome stability (Ackermann et al 2011, Nomura et al 2011). The FA pathway has been implicated in the repair of replication blocking lesions such as ICLs (Hiom 2010) and helps coordinate ICL repair via translesion synthesis (TLS) and homologous recombination (HR - a recombination event where nucleotide sequences are exchanged between two identical DNA strands) (Niedzwiedz et al 2004) (Knipscheer et al 2009) and mismatch repair (Williams et al 2011b). Defects in these processes could help explain why FA cells and patients are sensitive to ICLs.

FA gene/(alternative name)	Complex/Role	Chromosomal location	Protein size KDa	Prevalence in FA patients
FANCA	Core complex	16q24.3	160	65%
FANCB	Core complex	Xq22.31	95	2%
FANCC	Core complex	9q22.3	63	15%
FANCE	Core complex	6p21-22	60	2%
FANCF	Core complex	11p15	42	2%
FANCG	Core complex	9p13	68	10%
FANCL	Core complex	2p16.1	43	0.2%
FANCM	Core complex	14q21.3	250	0.2%
FANCD2	ID complex	3p25.3	155,162	2%
FANCI	ID complex	15q25-26	140,147	2%
FANCJ(BRIP1)	FANCJ/BRCA1 complex	17q22-24	140	2%
FANCD1(BRCA2)	BRCA1/BRCA2/PALB2 complex	13q12-13	380	2%
FANCN (PALB2)	BRCA1/BRCA2/PALB2 complex	16p12	140	2%
FANCO(RAD51C)	Homologous Recombination	17q25.1	42	0.4%
FANCP(SLX4)	Homologous Recombination	16p13.3	200	1.2%

Table 1. Currently identified proteins involved in the FA pathway (Kitao and Takata 2011). The above table shows alphabetically the proteins implicated in FA and their role in the FA pathway. Their chromosomal location, molecular weights and prevalence in FA patients are also shown.

DNA double strand breaks are repaired either by HR or by non-homologous end joining (NHEJ) (Kass and Jasin 2010). It has been suggested that the main function of FA proteins is to prevent the toxic engagement of the NHEJ repair proteins and to allow the homologous recombination dependent crosslink repair (Adamo et al 2010, Pace et al 2010). These 15 FANC proteins have been classified into three groups on the basis of their role in the monoubiquitination of FANCD2 and FANCI. Ubiquitination of these proteins is used as a marker for activation of the FA pathway (Figure 1).

Group 1: The FA core complex – It is composed of at least eight proteins: FANC – A, B, C, E, F, G, L and M. This complex acts as an E3 ubiquitin ligase which monoubiquitinates, and thus activates, the ID complex. It also includes FAAP20 (Ali et al 2012), FAAP24 (Ciccia et al 2007) and FAAP100 (Ling et al 2007), which are binding partners of FANCA, FANCM and FANCL respectively. The core complex also includes the proteins HES1 (Tremblay et al 2008) and MHF (Yan et al 2010). Although required for the monoubiquitination of the ID complex, it is likely that the proteins of the core complex have additional roles in DNA damage response (Matsushita et al 2005).

Group 2: The ID complex – FANCD2 and FANCI. Monoubiquitination of these proteins is a critical step in the response to ICLs by the FA pathway. This complex is also associated with FAN1 nuclease. Monoubiquitinated FANCD2–FANCI recruits

FAN1 to sites of damage, where FAN1 performs nucleolytic processing during ICL repair (Huang and D'Andrea 2010).

Group 3: The homologous recombination repair effector proteins – FANCD1, FANCJ, FANCN, FANCO and FANCP. These downstream proteins have been implicated in hereditary susceptibility to breast cancer, particularly FANCD1 (BRCA2), FANCJ (BRIP1/BACH1) and FANCN (PALB2). Inactivation of these genes causes FA when both alleles are mutated (homozygous) and predisposes to breast cancer when one allele is mutated (heterozygous). Moreover, the clinical presentation of FA due to biallelic FANCD1 or FANCN inactivation are much more severe than for other types of FA. They are associated with homologous recombination. Recently, it was shown that Rad51 binds ICL stalled forks independently of the ID complex and before the formation of double strand breaks (Long et al 2011). BRCA1, has recently been suggested to have an accessory role in mediating optimal FANCD2 foci formation, although, the loss of BRCA1 only decreases FANCD2 foci and does not ablate it completely (Bunting et al 2012). BRCA1 is also present in the downstream protein foci, is known to exist in multiple complexes to regulate various cellular processes, including checkpoint activation and HR to maintain genome integrity (Greenberg et al 2006).

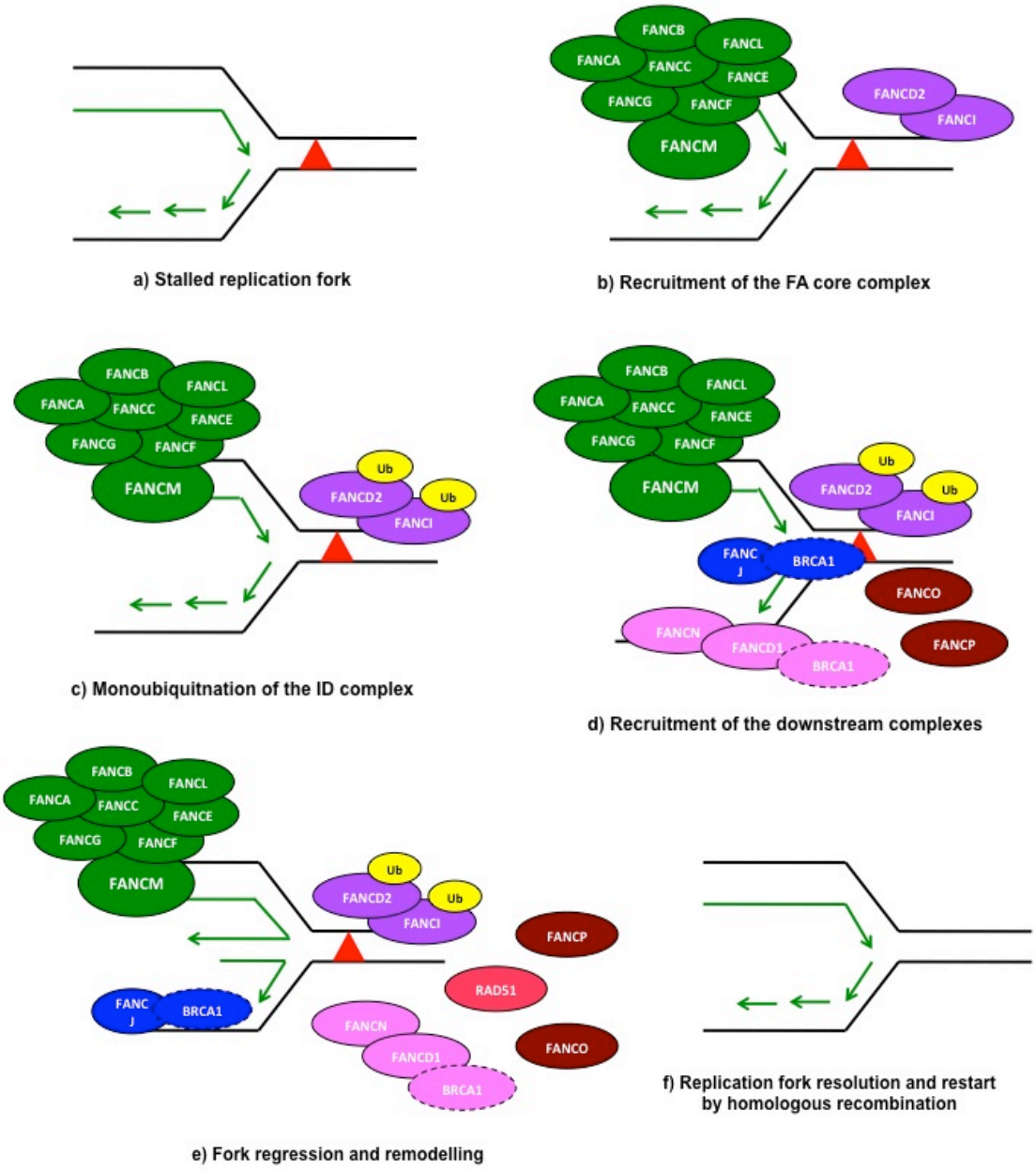


Figure 1. Schematic representation of the FA pathway. Stalled replication forks are recognized by FANCM, which recruits the FA core complex. The FA core complex monoubiquitinates the FANCD2 and FANCI complex. This results in their interaction with a number of downstream proteins, FANCD1, FANCN, FANCJ, FANCO, FANCP and facilitates downstream repair processes.

With a few exceptions, most of the FA proteins lack identifiable domains and motifs to suggest what biological activities they may have. The most conserved of the FA proteins are FANCM, SLX4 and FANCJ. Such an exquisite conservation, reflects the importance of these proteins in DNA repair and the possibility of these proteins having functions outside of ICL repair (Garner and Smogorzewska 2011a).

In response to ICLs, cells elicit the S-phase checkpoint arrest, which is mediated by ATR (Ataxia telangectasia mutated and Rad3 related) and its downstream kinase Chk1 (Pichierri and Rosselli 2004). Activated ATR-Chk1 kinase phosphorylates many FA proteins, e.g. phosphorylation of FANCI is essential for FANCD2 monoubiquitination (Ishiai et al 2008). In mammalian cells, the phosphorylation of FANCD2 at T691, S717 and S331 is required for potent FANCD2 monoubiquitination and for resistance to DNA cross-linking agents (Ho et al 2006, Zhi et al 2009). It has also been shown that the phosphorylation of FANCA at S1449, FANCG at S7 of FANCE at T346 and S374 is important for the cellular resistance to crosslinking agents (Collins et al 2009, Qiao et al 2004, Wang 2007). In particular, the phosphorylation of FANCM leads to the assembly of the FA core complex (Kim et al 2008).

FANCM is a DNA translocase, which can bind and move the branch point of forked DNA structures similar to replication or repair intermediates (Xue et al 2008, Meetei et al 2005). FANCM forms a conserved DNA remodelling holoenzyme with the MHF1 and MHF2 together known as MHF (major histone fold heterodimer) (Yan et al 2010) and associated factor FAAP24. Together, they regulate several independent aspects of the DNA damage response (Ciccia et al 2007). FANCM and FAAP24 interact with the checkpoint protein HCLK2 and facilitate DNA damage signalling which is mediated by ATR (Collis et al 2008). FANCM also associates with the Bloom's complex (BLM-TopoIIIα-RMI1-RMI2) through direct interaction with the RMI1 and TopoIIIα subunits (Meetei et al 2003, Deans and West 2009). FANCM and FAAP24 recruit the FA core complex to chromatin (Kim et al 2008), through a direct FANCM and FANCF interaction (Deans and West 2009). Replication blocking lesions activate the FA core complex. The core complex, acts as an E3 ubiquitin ligase, where FANCL is the catalytic subunit, (Seki et al 2007) which monoubiquitinates the ID complex – FANCD2 at lys561 and FANCI at lys563 (McCabe et al 2009). It is currently not known how the other proteins of the FA core complex assist or regulate FANCL ubiquitin ligase activity in response to DNA damage. Recently, it has been proposed that FANCD2 monoubiquitylation may occur after it binds to the damaged DNA (Sato et al 2012). The crystal structure of the ID complex was elucidated (Joo et al 2011) and it was found that in the ID complex, the monoubiquitylation site of FANCD2 is buried between the FANCI and FANCD2 interface. To explain how this buried site becomes accessible for monoubiquitylation, it was proposed that a conformational change of the ID complex occurs which relocates the FANCD2 monoubiquitylation site on the surface of the complex (Joo et al 2011) (Figure 2).

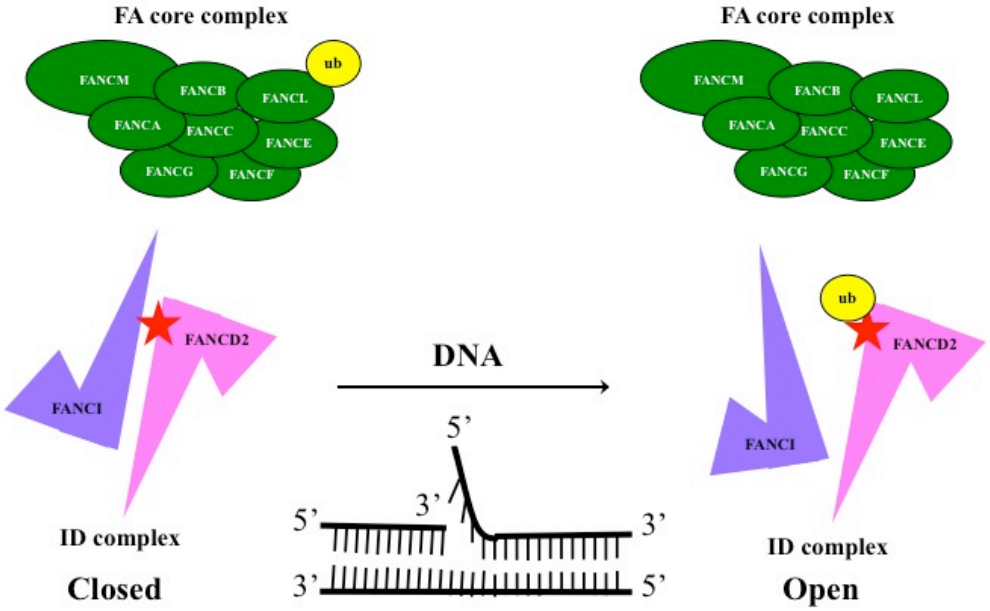


Figure 2. Proposed model for the role of branched DNA in FANCD2 monoubiquitylation (Sato et al 2012). The monoubiquitylation site of FANCD2 (represented by a star) is located near the interface with FANCI. As such, the E3 ligase FANCL is not able to access the FANCD2 monoubiquitylation site in the ID complex (closed conformation). Branched DNA binds to FANCI and is able to induce a conformational change of the ID complex, which exposes the FANCD2 monoubiquitylation site for ubiquitylation by FANCL (open conformation).

Although, currently, it is known that the FA core complex monoubiquitylates FANCD2 and that this monoubiquitylation is required to stabilise the ID complex on chromatin, it has been proposed that the FA core complex also functions in the chromatin targeting of the ID complex which is then followed by FANCD2 monoubiquitylation (Sato et al 2012). This monoubiquitylation of the ID complex may help to prevent the dissociation of the ID complex from the damaged chromatin (Sato et al 2012).

Recently, it has been shown that RAD18 also contributes in the regulation of FANCD2 monoubiquitination (Geng et al 2010, Williams et al 2011a). RAD18 is an E3 ligase and in response to DNA damage, RAD18 monoubiquitinates PCNA which leads to the recruitment of TLS polymerases, amongst other functions (Song et al 2010). In response to different genotoxic damage, e.g. that induced by camptothecin and MMC, the monoubiquitination of FANCD2 is dependent on RAD18 (Palle and Vaziri 2011, Williams et al 2011a). It is still not fully understood how RAD18 participates and regulates the FA pathway.

It has also been shown that MSH2 interacts with FANCD2 and is also needed for the monoubiquitylation and chromatin loading of FANCD2 and FANCI (Williams et al 2011b). The loss of MSH2 only decreases FANCD2-FANCI foci formation and does not ablate it completely. It has been suggested that MSH2 plays a crucial role in the detection of ICLs and in the early signaling events, which lead to the activation of the FA pathway (Williams et al 2011b).

Phosphorylation and monoubiquitination of the ID complex act as a crucial switch, which signals initiation of the FA pathway. This leads to recruitment of various DNA repair factors essential for homologous recombinational repair, e.g. FAN1, BRCA1, FANCD1, FANCO, FANCP, FANCJ and FANCN, they are involved in the repair of double-strand breaks generated during replication-coupled ICL repair and promote the restart of the stalled replication fork.

Deubiquitination of FANCD2 is an equally important event and it is under the control of USP1 (Kim et al 2009, Nijman et al 2005) in association with UAF1 (Cohn et al 2007). USP1 binds FANCI directly via its SUMO like domain, which associates with the SUMO like domain-interacting motif on FANCI (Murai et al 2011). Removal of the ubiquitin bound to FANCD2 and FANCI is necessary for completion of the DNA repair process and ICL tolerance (Kim et al 2009).

FAN1 (FANCD2-associated nuclease 1) was discovered independently by five groups (Kratz et al 2010, Liu et al 2010, MacKay et al 2010, Shereda et al 2010, Smogorzewska et al 2010). It is necessary for chromosome stability and cellular tolerance to crosslinking agents. ICL treated cells show FAN1 foci, which co-localise with the monoubiquitinated FANCD2. FAN1 contains a ubiquitin binding domain (UBZ), which is necessary for interaction with FANCD2 and for targeting of FAN1 to DNA repair sites (Kratz et al 2010, Liu et al 2010, MacKay et al 2010, Shereda et al 2010). FAN1 exhibits $5' \rightarrow 3'$ exonuclease activities and participates in DNA repair mechanisms dependent on homologous recombination (Kratz et al 2010, Liu et al 2010, MacKay et al 2010, Smogorzewska et al 2010). It has been suggested that FAN1 does not function with FA proteins in the resolution of endogenous replication obstacles (Yoshikiyo et al 2010). It is still not clear how FAN1 acts during repair of DNA damage induced by crosslinking agents.

It is known that FANCJ interacts with BRCA1 and unwinds DNA structures that block replication forks and facilitates checkpoint signalling (Bridge et al 2005, Cantor et al 2001, Gupta et al 2005, Hiom 2010). FANCJ also forms a complex with BLM, and the two helicases can unwind DNA substrates synergistically (Suhasini et al 2011). Since BLM is unstable in the absence of FANCJ, the overlap of symptoms in FANCJ and BLM patients may be linked to a deficiency in BLM protein (Suhasini et al 2011).

FANCD1 (BRCA2) and FANCN are key regulators of homologous recombination. FANCD1, the product of the breast cancer susceptibility gene, provides a structural platform for the fine regulation of the strand exchange protein RAD51 (Holloman 2011).

The RAD51 protein is a recombinase, which is important for the homologous recombinational repair of DSBs and ICLs (Long et al 2011). The RAD51 paralogs RAD51B, RAD51C and RAD51D are known to regulate HR in coordination with other DNA repair factors e.g. XRCC2 and XRCC3 (Somyajit et al 2010). Studies have shown that FANCO/RAD51C exists in two major complexes, RAD51B/RAD51C/RAD51D/XRCC2 and the RAD51C/XRCC3. Evidence from various studies shows that FANCO participates in the initial and late stages of HR (Somyajit et al 2010). Cells lacking RAD51C have been shown to have reduced

RAD51 foci after exposure to ionising radiation (IR), decreased DSB repair by HR, and elevated chromosomal aberrations. It has also been shown that FANCO participates in DNA damage signaling by facilitating the phosphorylation of the checkpoint effector kinase CHK2 (Badie et al 2009).

BRCA1 and FANCD1 are two DNA repair factors, which are required for HR-mediated DSB repair in both mitotic and meiotic cells (Scully et al 1997). These two proteins are known to co-localize with RAD51 in distinct nuclear foci in the S and G2 phase of cell cycle. This interaction of BRCA1 and BRCA2/FANCD1 with RAD51 was identified in a complex, known as the BRCA1-BRCA2-RAD51 complex (Chen et al 1998). The BRCA1-BRCA2-RAD51 complex localizes to the sites of stalled replication forks, which suggests the involvement of BRCA1 and BRCA2 in the HR-mediated repair of DNA lesions that arise as a result of replication fork stalling (Chen et al 1998). RAD51 recombinase directly interacts with BRCA2 and this association plays an important role in the assembly of RAD51 to the replication protein A-coated single stranded DNA to facilitate HR (Jensen et al 2010).

FANCN (PALB2) was identified as a FA protein by searching for novel interacting partners of endogenous BRCA2 (Xia et al 2006). The protein is associated with approximately 50% of endogenous BRCA2 and is critical for the chromatin localization and recruitment of BRCA2/FANCD1 to the sites of DNA damage (Xia et al 2006). BRCA1 has been implicated in transcriptional regulation of many genes and in cellular redox regulation (Bae et al 2004) (Saha et al 2009). It is known to be important for HR. FANCN and BRCA1 are known to coexist in an endogenous protein complex (Chen et al 1998). It has been established that PALB2 is able to physically and functionally link BRCA1 and BRCA2/FANCD1 to form a BRCA complex (Zhang et al 2009). The integrity of this BRCA complex is essential for the initiation of HR and for the suppression of FA (Sy et al 2009).

SLX4/FANCP is scaffold protein, which connects various nucleases to the FA pathway (Kim et al 2011, Stoepker et al 2011). FANCP is necessary for homologous recombinational repair (Munoz et al 2009). It has been proposed that FANCP can act as a regulatory platform associated with multiple structure specific endonucleases, e.g. SLX1, XPF-ERCC1 and MUS81-EME1 (Munoz et al 2009, Svendsen et al 2009). It is still not clear how FANCP coordinates the activities of these structure specific nucleases. FANCP contains multiple protein-protein interaction modules (Svendsen et al 2009). The human SLX1–FANCP complex exhibits 5'-flap endonuclease activity and resolves Holliday junctions (Munoz et al 2009, Svendsen et al 2009). SLX1–FANCP is a Holliday junction resolvase as it is able to produce directly nicked duplexes (Fekairi et al 2009). SLX4 plays an important role in the repair of DNA lesions that arise spontaneously (Yamamoto et al 2011). FANCP is connected to the FA pathway by its UBZ domain, which is necessary for tolerance to crosslinking agents. FANCP forms ICL-induced foci in cells and associates with FANCD2. The recruitment of FANCP at DNA repair sites depends on the integrity of its UBZ domain and on the FA core complex (Yamamoto et al 2011). Also, it has been shown that FANCC and FANCP double mutants are more sensitive to crosslinking agents than either single mutant, this suggests that FANCP and the FA core complex have also non-epistatic roles in ICL repair (Yamamoto et al 2011).

How exactly the FA pathway promotes HR is still unclear. Recent studies have shown that the Fanconi anaemia pathway facilitates replication coupled homologous recombination in mammalian cells (Nakanishi et al 2011). It has also been shown that DNA replication had little impact on recombination repair induced by a DSB, whereas, ICL-induced homologous recombination was greatly stimulated when coupled to replication and was also strongly dependent on the integrity of the FA pathway (Nakanishi et al 2011).

Broadly speaking, the FA pathway is a window into evolution. Only vertebrates have a fully complemented FA pathway. Lower eukaryotes and prokaryotes have only a few or none of the homologs. Increase in genome complexity, advent of multicellularity and tissue differentiation indicates the need for many complex, coordinated and regulated interactions needed to maintain genomic stability, prevent DNA damage and to promote repair. This is also correlated with increase in the number of FA proteins. Presence of a FA protein in a particular organism or at certain stage of evolution suggests the need to address the requirement to maintain an increasingly complex genome.

1.1 FANCJ

To further explore the FA pathway and help gain a mechanistic understanding of ICL repair, we decided to elucidate the role of FANCJ in ICL repair. FANCJ is an ATP-dependent 5'→3' DNA helicase (Wu and Brosh 2009). Initially, it was identified as interacting partner of BRCA1 (Breast Cancer associated tumor suppressor 1). It was originally named BACH1 (BRCA1-associated C-terminal Helicase1) but was later renamed BRIP1 (BRCA1 Interacting Protein C-terminal helicase1) because of the presence of another transcription factor of the same name BACH1 (Cantor et al 2001). FANCJ is a 140 KDa protein conserved down to *C. elegans (dog-1)*. It belongs to the Q-motif DEAH box (Asp-Glu-Ala-His) helicase family. Helicases belonging to the DEAH family are processive, meaning that the protein utilizes its motor ATPase/translocase function to continuously and directionally move or translocate along nucleic acids and displaces the paired strands or proteins in its path (Pyle 2008, Sommers et al 2009). The Q-motif (G-F-X-X-P-X-P-I-Q) was first identified in DEAD box RNA helicases. It is located approximately 17 amino acids upstream of the Walker A box and consists of a nine amino acid sequence consisting of a glutamine (Q) residue (Tanner et al 2003). Site-specific mutagenesis studies proved that the Q motif controls ATP binding and hydrolysis and that it is important for cell viability (Tanner et al 2003). Aromatic residues have been proposed to assist in the hydrophobic stacking (Tanner 2003). It is known that the Q motif is responsible for sensing the nucleotide state(s) and establishing a stable interaction of the Walker A box with other helicase motifs, and this stabilization is required for catalytic competence (Strohmeier et al 2011). FANCJ has a core helicase domain, N-terminal nuclear localization signal sequence, a conserved Fe-S cluster (Rudolf et al 2006) and BRCA1 interacting domain (Cantor et al 2001) (Figure 3). Fe-S clusters are present in helicases engaged in DNA damage response e.g. base excision repair glycosylases (Saporito et al 1989). It has been proposed that the Fe-S cluster helps in locating sites of DNA damage (Lukianova and David 2005).

Germline mutations that abolish the helicase activity of FANCJ were shown to result in early onset breast cancer (Lalloo et al 2006, Cantor et al 2004). The presence of mutations in patients within the FA complementation group J implicates FANCJ in ICL repair (Levitus et al 2005, Litman et al 2005). It is the helicase function of FANCJ that is important in maintaining genome stability and in preventing disease. Cells expressing a helicase defective K52R mutant FANCJ protein exhibit enhanced sensitivity to ICL agents and an increased number of chromosome aberrations, which is characteristic of Fanconi anemia (Cantor et al 2001, Bridge et al 2005). Truncated forms of FANCJ expressed in FA-J patients are defective in FANCJ-mediated DNA unwinding (Cantor et al 2004, London et al 2008). A Q25A mutation in the Q motif was shown to abolish its ability to complement cisplatin or telomestatin sensitivity in a FANCJ null cell line and exerted a dominant negative effect. Biochemical characterization of the mutated FANCJ Q25A protein has shown that the mutation disables FANCJ helicase activity and its ability to disrupt protein-DNA interactions. Mutated FANCJ Q25A also showed impaired DNA binding and ATPase activity (Wu et al 2012).

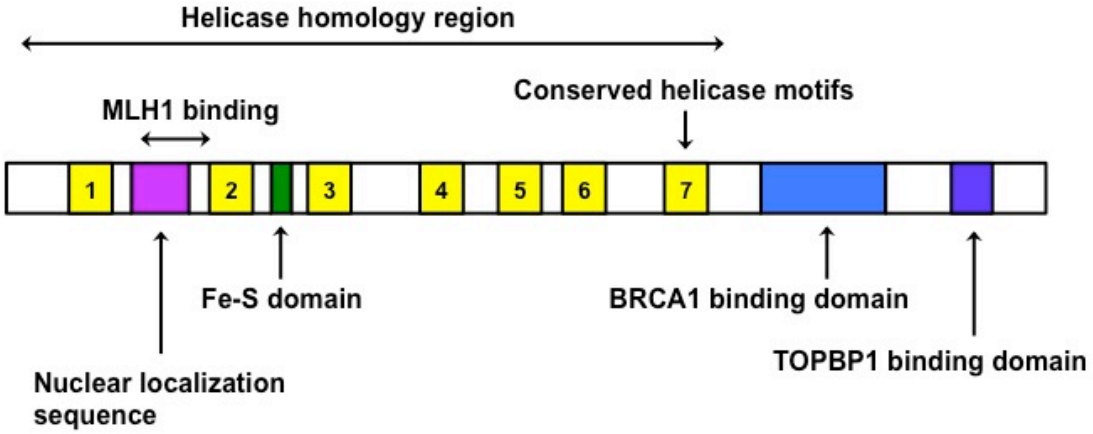


Figure 3. Schematic representation of the FANCJ helicase (Wu and Brosh 2009)**.** The human FANCJ protein contains 1249 amino acids. The nuclear localization sequence is indicated by pink box, the position of the iron-sulfur domain is indicated by a green and BRCA1 and TopBP1 interaction domains are indicated by blue and purple boxes respectively. MLH1 binding domain is indicated above the protein. Not to scale.

FANCJ has been suggested to function during DNA replication as it is implicated in the repair of ICLs that stall replication forks (Shen et al 2009) and it is known to become phosphorylated and undergoes relocalization to chromatin during S-phase (Cantor et al 2001, Yu et al 2003). It is also suggested that FANCJ is required for the timely progression of cells through S-phase (Kumaraswamy and Shiekhattar 2007). Exposure of cells lacking FANCJ to DNA damage results in incomplete replication and accumulation at late S/G2 phase. FANCJ helicase is able to unwind DNA substrates that resemble replication fork intermediates (Gupta et al 2005).

In response to DNA damage, FANCJ colocalizes with Replication Protein A (RPA) (Gupta et al 2007), which acts as an assisting factor for the unwinding function of FANCJ. Since FANCD2 monoubquitination is intact in FANCJ deficient cells, it appears to act downstream, or partly independent of, the ID complex. Its role in the repair of ICL has not been defined. It is known to interact with MutLα, and the authors suggest that this interaction is required for the repair of ICLs (Peng et al 2007). It also interacts with Bloom's helicase (Suhasini and Brosh 2011) that is required in the dissolution of Holliday junctions arising as intermediates of homologous recombination. FANCJ can destabilize RAD51 nucleoprotein filaments, and as such, may have an anti-recombination function which reverses the formation of recombination intermediates formed in the early stages of HR (Sommers et al 2009). It is also involved in the repair of DNA double-strand breaks by HR in a manner that depends on its association with BRCA1 (Wu and Brosh 2009). FANCJ is able to unwind a variety of DNA substrates and might have the ability to sense DNA damage in either strand of the duplex and facilitate DNA damage processing (Wu and Brosh 2009). It is also able to unwind G4 DNA in a reaction dependent on adenosine triphospate (ATP) hydrolysis (Wu et al 2008). FANCJ has been linked to deletions in G-rich sequences (London et al 2008). It has been shown that FANCJ acts with WRN and BLM helicases and that FANCJ and REV1 also act together to defend epigenetic stability by ensuring continuous replication through G-quadruplex regions in the genome. FANCJ and BLM have been shown to interact and colocalize to the sites of stalled replication forks (Suhasini et al 2011). It has been suggested that WRN and BLM access a G-quadruplex in the direction opposite to FANCJ, allowing them to

collaborate in facilitating DNA replication through the G-quadruplex (Sarkies et al 2012).

FANCJ also provides another link between FA proteins and checkpoint signaling. FANCJ is known to interact with TopBP1 (Gong et al 2010). This interaction leads to the phosphorylation of FANCJ at Thr 1133 and is mediated by the very C-terminal BRCT repeats 7 and 8 of TopBP1, which then leads to a conformational modification (Leung et al 2011). In cells exposed to hydroxyurea, the helicase activity of FANCJ and its interaction with TopBP1 are both required for the accumulation of RPA onto chromatin. This eventually promotes the assembly of the ATR signalling complex and the phosphorylation of its downstream effector protein Chk1 (Gong et al 2010). Thus, FANCJ helps to facilitate ATR activation by unwinding the DNA at stalled replication forks.

The interaction of FANCJ with diverse proteins involved in signaling DNA damage and proteins which belong to different DNA repair pathways suggests that it may be a constituent of diverse repair complexes formed in response to different kinds of DNA damage. It is important to understand how proteins from various pathways can cross-talk with FANCJ, how this leads to the identification and eventual repair of different kinds of DNA damage and the implications of disruption(s) in this possible cross-talk.

1.2 AIMS OF THE PROJECT

Thus, in order to study the functions and interactions of FANCJ and elucidate its role in ICL repair, the aims of my project were to endogenously express StrepII/FLAG tagged FANCJ and to optimize the immunoprecipitation protocol for the purification of the FANCJ associated protein complexes.

Here, I used the avian B lymphocyte cell line DT40 as a model system. DT40 is an avian leucosis virus transformed chicken B-lymphocyte (Arakawa and Buerstedde 2004), which exhibits high ratios of targeted to random integration with transfected DNA constructs. This efficient targeted integration makes them a convenient for generating cell lines with different modifications (Buerstedde and Takeda 1991). Another advantage of using this cell line is the high level of conservation of the FA pathway between human and avian systems. These advantages, as such, make the DT40 cell line an ideal model to study the FA pathway.

These cell lines are important tools that will be utilized to define and characterize the role of FA pathway and systematically investigate the possible multiprotein complexes formed by FANCJ and define an interactome, which will provide information both about the functions of individual proteins and the functions of the complexes they interact with.

Chapter 2: MATERIALS AND METHODS

2.1 MATERIALS

See Appendix

2.2 METHODS

2.2.1 Generation of C-terminally Strep and FLAG tagged FANCJ targeting vectors

To generate a targeting vector, the genomic locus of the protein of interest from species *Gallus gallus* was mapped and amplified by PCR. Part of the genomic sequence outside the C-terminus was also mapped and amplified by PCR. This DNA was then used to generate targeting constructs allowing for insertion of the Strep/FLAG tag coding sequence after the last exon (C-terminus) of genomic locus of interest, namely FANCJ. The Strep/FLAG tagged FANCJ targeting vector was generated by Dr. Andrew Blackford.

A C-terminus targeting vector (Figure 2.2.1) contains, a 5' arm – comprising part of the sequence of the last exon with the stop codon removed from the gene of interest. The tag coding sequence with a stop codon at the end follows this. A resistance cassette/selection marker – antibiotic resistance and its β-actin promoter flanked by LoxP sites - is present afterwards and a 3' arm that comprises part of the genomic sequence beyond the last exon of the relevant genomic locus completes the targeting vector. These DNA sequences were cloned into a pBluescriptKS+/- backbone.

Each targeting vector contains a resistance cassette flanked by LoxP sites, which allows for initial selection of the targeted cells. When the targeting vector with its resistance cassette is integrated into the genomic locus, the cell line is said to be 'loxed'. After targeted integration has been confirmed, the resistance cassette is removed. The genomic locus with the targeting construct - free of the resistance cassette - is said to be 'unloxed'. It is the tagged cell line free of the selection marker, which is used for further analysis.

The strategy to generate a SF-tagged cell line is shown in Figure 2.2.2.

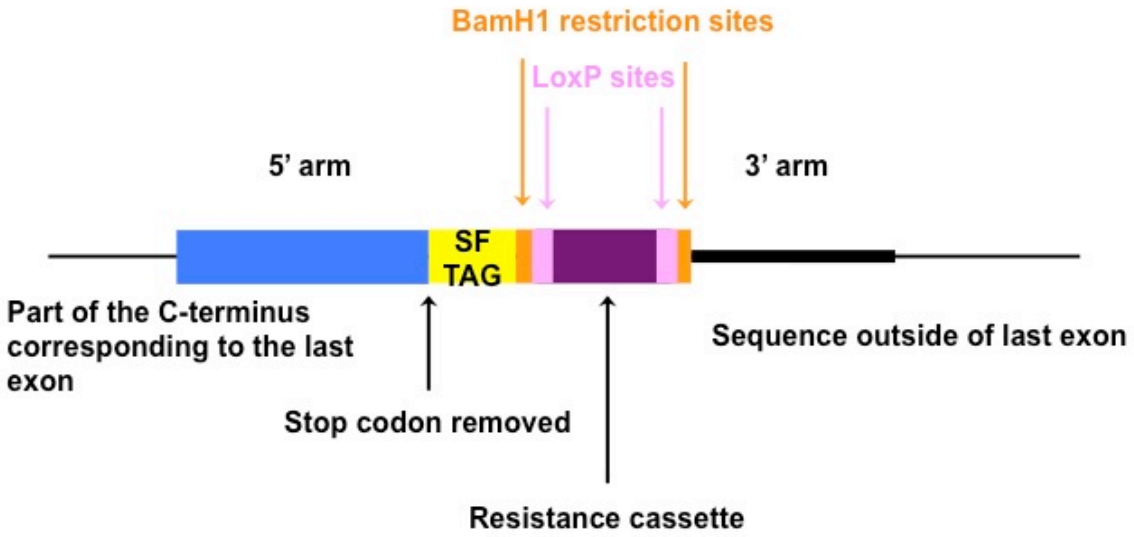


Figure 2.2.1: Cartoon representation of a linearized targeting vector. SF tag – 2x Strep and FLAG tag. Not to scale.

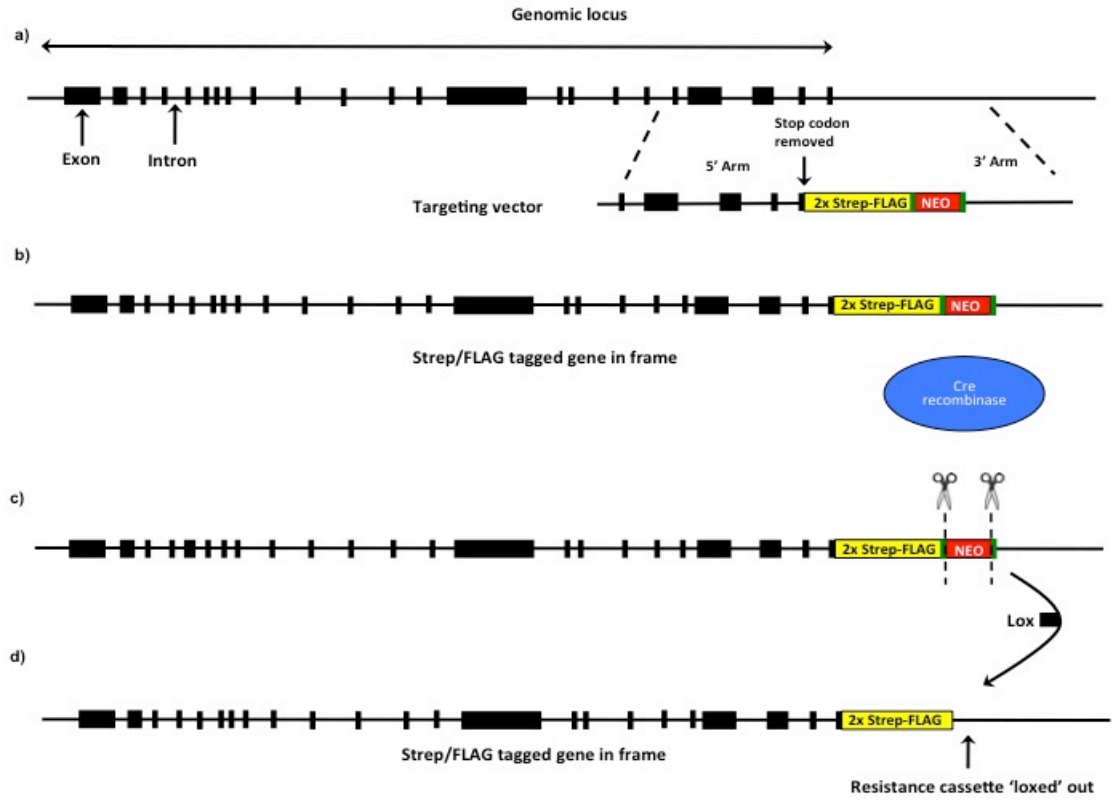


Figure 2.2.2. Strategy for generation of endogenously tagged proteins. a) Map showing the genomic locus of the protein of interest. **b)** A homologous recombination event will result in the last exon being extended with inframe sequence of the 2x Strep/FLAG tag coding sequence, followed by the stop codon, the loxed resistance cassette (Neomycin) and sequence from the 3' homology region. **c)** Unloxing the tagged genomic locus by Cre recombinase, which recognizes the LoxP sites flanking the resistance cassette (denoted by green lines) and excises it out of the genomic locus. **d)** In-frame unloxed tagged genomic locus. Black boxes represent positions of exons. Sizes of exons are not to scale.

2.2.2 Bacterial culture and generation of targeting vectors

2.2.2.1 Growth of transformed bacterial culture with C-terminally Strep and FLAG tagged FANCJ targeting vector with Neomycin resistance cassette.

The transformed bacterial stock (DH5α) with C-terminally Strep and FLAG tagged FANCJ targeting vector with Neomycin resistance cassette was used to inoculate an LB agar selection plate (Ampicillin) overnight. Several colonies were picked from this LB agar plate and transferred into 2 mL LB medium with appropriate antibiotic (Ampicillin). The culture was grown for 12 hours at 220 rpm at 37 ^{o}C in an orbital bacterial shaker. This bacterial culture was then transferred into 150 mL LB medium with appropriate antibiotic (Ampicillin). This culture was grown for 12 hours at 220 rpm at 37 ^{o}C in a bacterial shaker. This 150 mL culture (grown in Erlenmeyer flask) was then subject to plasmid extraction using the GenElute HP Endotoxin Free Maxiprep Kit. The quality of the eluted plasmid was assessed using a spectrophotometer. An eluted plasmid aliquot with minimum concentration of 500 ng/μL and an A_{260}/A_{280} ratio of 1.8 was used for subsequent downstream application.

2.2.2.2 Linearization of the eluted C-terminally Strep and FLAG tagged FANCJ targeting vector with Neomycin resistance cassette.

To prepare the aliquot for transfection, the C-terminally Strep and FLAG tagged FANCJ targeting vector with Neomycin resistance cassette was linearized. An aliquot of concentration 200 μg was subject to a 12-hour digestion with the restriction enzyme Not1. This digested aliquot was then purified and concentrated using Isopropanol and 70% Ethanol. The purified and linearized sample was re-suspended in MilliQ water. The quality of this purified and linearized plasmid was assessed using spectrophotometer. The purified plasmid aliquot with minimum concentration of 500 ng/μL and an A_{260}/A_{280} ratio of 1.8 was used for subsequent transfection to target the 1st FANCJ allele.

2.2.2.3 Recycling targeting vectors

In order to re-use the C-terminally Strep and FLAG tagged FANCJ targeting vector for a second round of stable targeted transfection in the generated heterozygous Strep and FLAG tagged FANCJ cell line, the Neomycin resistance cassette present was exchanged with Blasticidin resistance cassette. An aliquot of the targeting vector with Neomycin was subject to BamH1 restriction digest for 3 hours at 37 ^{o}C. The digested plasmid DNA was run on a 0.7% agarose gel and the targeting vector minus the resistance cassette was excised and purified using a Gel purification kit. This product was ligated with Blasticidin resistance cassette using T4 DNA ligase using Rapid ligation buffer at 37 ^{o}C for 1 hour, after which an aliquot of DH5α were transformed with the recycled targeting vector. The transformed DH5α were grown on LB agar selection plates (Ampicillin). Several colonies were picked from this LB agar plate after 12 hours and transferred into 2 mL LB medium with appropriate antibiotic (Ampicillin). The culture was grown for 12 hours at 220 rpm at 37 ^{o}C in a bacterial shaker. These cultures were subject to plasmid extraction using the GeneJet Plasmid miniprep kit. The recycled targeting vector was subject to BamH1 restriction digest for 3 hours at 37 ^{o}C. The digested plasmid DNA was run on a 0.7% agarose gel. The

bacterial colony corresponding to the restriction digest that produced the predicted restriction fragments was expanded and the plasmid DNA purified using GenElute HP Endotoxin Free Maxiprep Kit. The eluted plasmid was used as before for subsequent transfection to target the 2nd FANCJ allele.

2.2.2.4 Transformation of bacteria

A 100μL aliquot of competent DH5α was thawed on ice. 2μL plasmid in H_2O was added and left on ice for 15 minutes. This was followed by 45 second heat shock at 42 ^oC. Bacteria were returned to ice for 5 minutes. After cooling, 300μL of LB medium was added and the bacteria incubated for 1 hour at 37 ^oC. 100μL of bacterial suspension was plated on LB agar with appropriate selection (Ampicillin).

2.2.2.5 Bacterial stock

Colonies from the transformed bacteria containing the recycled C-terminally Strep and FLAG tagged FANCJ targeting vector with Blasticidin resistance cassette were picked from the LB agar selection plate and transferred into 2 mL LB medium with appropriate antibiotic (Ampicillin). The culture was grown for 12 hours at 220 rpm at 37 ^oC in a bacterial shaker. The bacterial suspension was then aliquoted and thoroughly mixed with equal amounts of 100% glycerol. Aliquots were frozen in dry ice and stored for further use at -80 ^oC.

2.2.3 Cell lines and generation of C-terminally Strep and FLAG tagged FANCJ cell line

2.2.3.1 Cell lines and cell culture

The DT40 cell lines were used.

DT40 (wild-type and knock-in) cell lines were grown in suspension. They were maintained in Roswell Park Memorial Institute (RPMI) medium (Invitrogen), supplemented with 8% foetal bovine serum (PAA laboratories), 2% chicken serum (Sigma Aldrich), 100μg/mL of penicillin (PAA laboratories), 100μg/mL streptomycin (PAA laboratories) and 0.1% β-Mercaptoethanol (Sigma Aldrich); at 38 ^oC, 5% CO_2.

The cells were kept at a density of 0.5 x 10^6 per mL. Confluent cells were split by removing the calculated volume of culture medium followed by addition of calculated volume of the prepared fresh medium.

2.2.3.2 Stable targeted transfection (Gene targeting) of the 1st FANCJ allele

Stable targeted transfection of the C-terminally Strep and FLAG tagged FANCJ targeting vector with Neomycin resistance cassette into the 1st FANCJ allele was carried out using 20 x 10^6 cells and 40μg linearized targeting vector DNA per transfection. Transfection was carried out using a BioRad Gene Pulser II, under the following conditions: 300V, 600μF. This was followed by antibiotic selection where, in brief, cells were diluted in medium containing the antibiotic – neomycin (2mg/mL) and plated in 96 well plates. They were incubated for 10 - 15 days. Positive colonies were selected and grown in 24 well plates for 1-2 days before each was split into two.

One set was frozen; the other set was harvested for genomic DNA and screened for targeted integration.

2.2.3.3 Genomic DNA (gDNA) extraction

2 x 10^6 cells were used for gDNA extraction, using the Puregene DNA Isolation kit according to manufacturer instruction. The extracted gDNA was used to screen generated clones for targeted integration.

2.2.3.4 PCR screen for targeted integration of the C-terminally Strep and FLAG targeting vector with Neomycin resistance cassette.

To screen for stable targeted integration of the vector into the proper genomic locus, a PCR-based screening system was developed by Dr. Jadwiga Nieminusczy. A primer annealing 296 base pairs upstream of the 5' arm of the targeting construct was used for this PCR together with a primer annealing within the Neomycin resistance cassette. Positive targeted integration was identified by the presence of a band of calculated size. PCR was carried out using Marathon polymerase (A&A Biotechnology), which is a mixture of two thermostable DNA polymerases (Taq – *Thermus aquaticus* and Pwo – *Pyrococcus woesei*) and thermostable UTPase. Such an enzyme composition enables the efficient synthesis of long amplicons. The PCR conditions were:

Denaturation – 94^o C for 30 sec
Annealing – 60^o C for 60 sec
Elongation – 68^o C for 3 min 30 sec (1kb/min) (Number of cycles- 40)

Primers

Primer name	**Sequence**
FANCJ 5'	5'-ACGCGTCGACGTTCAGCATACTGCAGT-3'
NEO	5'-CCGCTTCCTCGTGCTTTACGGTATCG-3'

2.2.3.5 Stable targeted transfection (Gene targeting) of the 2nd FANCJ allele

Stable targeted transfection of the C-terminally Strep and FLAG tagged FANCJ targeting vector with Blasticidin resistance cassette into the 2nd FANCJ allele was carried out using the protocol as described before. The generated colonies were screened for targeted integration.

2.2.3.6 PCR screen for targeted integration of the C-terminally Strep and FLAG targeting vector with Blasticidin resistance cassette.

Stable targeted integration of the vector into the proper genomic locus was screened using the PCR-based screening system described before. A primer annealing 296 base pairs upstream of the 5' arm of the targeting construct was used for this PCR together with a primer annealing within the Blasticidin resistance cassette. Positive targeted integration was identified by the presence of a band of calculated size.

Primers

Primer name	Sequence
FANCJ 5'	5'-ACGCGTCGACGTTCAGCATACTGCAGT-3'
BSR	5'-AGTGATGATGAGGCTACTGCTCAC-3'

2.2.3.7 Transient transfection

Once stable targeted integration of both the FANCJ alleles was confirmed, the selection markers were removed in order to minimize the modification to the genomic locus – this is known as 'unloxing'. To free the cell lines of the selection markers, the stable double 'knock-in' cell lines were subject to transient transfection using Amaxa Biosystems – Nucleofector II under the following conditions: 250V, 950μF. 5 x 10^6 cells and 15μg of Cre recombinase plasmid DNA (used to excise resistance cassette(s)) were used per transfection. Using electroporation solution, cells were nucleofected using programme B-23, which is designed for transient transfection. It changes the membrane potential of the cells and allows for the internalization of the plasmid DNA. After transfection, cells were diluted and plated at 1 cell/well, 2 cells/well and 4 cells/well. Single colonies were selected and grown in duplicate. Loss of resistance was confirmed by growing one set of clones in selection medium containing neomycin (2mg/mL) and Blasticidin (20μg/mL). Successful selection marker removal was demonstrated by death of transfected cells. The corresponding clone was taken from the duplicate plate, grown in an antibiotic free medium, expanded and used for further analysis.

2.2.3.8 Cell stocks

The generated heterozygous, homozygous and homozygous unloxed C-terminally Strep and FLAG tagged FANCJ cell lines were aliquoted and frozen in a cell freezing medium and stored for further use at the -80 ^{o}C ultra-freezer and the liquid nitrogen cell storage tank -196 ^{o}C.

2.2.3.9 Confirmation of in frame Strep/FLAG tag integration into both the FANCJ alleles

A PCR strategy was developed to confirm that both the FANCJ alleles had Strep and FLAG (SF) tagged. A primer pair annealing 1.9 kilo base pairs (kbp) upstream and 1.1 kbp downstream of the integrated Strep/FLAG tag in the FANCJ genomic locus would generate a PCR product of calculated size (3 kbp) in wild type FANCJ locus whereas the integrated in frame Strep/FLAG-tagged FANCJ locus would generate a PCR product extended by the 150 bps (3.1 kbp) of the Strep/FLAG-tag using the same primer pair. PCR was carried out using Marathon polymerase (A&A Biotechnology). The PCR conditions were:

Denaturation – 94^{o} C for 30 sec
Annealing – 60^{o} C for 60 sec
Elongation – 68^{o} C for 3 min 30 sec (1kb/min) (Number of cycles - 40)

Primers

Primer name	Sequence
FANCJ 5'	5'-ACGCTTCTTTTCTTCCTCCCCCA-3'
FANCJ 3'	5'-CCCCCTCTCCCACCTCCTCCT-3'

2.2.3.10 EcoR1 and BamH1 double digestion of the generated PCR products

The generated PCR products were subject to an EcoR1 and BamH1 double digest for 2 hours at 37 ^{o}C. On a 0.7% agarose gel, the Wild type and SF-tagged FANCJ cell lines 1 and 2 showed the predicted restriction fragments of 1.9 kb, 1.1 kb and 150 bp. The 150 bp restriction digest product was not visualized possibly due to the small size of the fragment.

2.2.3.10 Cloning of the generated PCR product from SF-tagged FANCJ cell line 1 into pCR 2.1-Topo cloning vector

In order to sequence the C-terminus of FANCJ genomic locus, the generated uncut PCR product from Strep/FLAG-tagged FANCJ cell line 1 was cloned into pCR 2.1-Topo cloning vector. To set up the cloning reaction, 4μL of the generated PCR product was mixed with 1μL of the salt solution and 1μL of the Topo vector provided. This cloning reaction was incubated at room temperature for 5 minutes and then kept on ice.

A 100μL aliquot of competent DH5α was thawed on ice. 6μL of the cloning reaction was added to the DH5α and left on ice for 15 minutes. The DH5α were heat shocked at 42 ^{o}C for 30 seconds. Bacteria were returned to ice for 5 minutes. After cooling, 250μL of SOC medium was added and the bacteria incubated for 1 hour at 37 ^{o}C. 100μL of bacterial suspension was plated on LB agar with appropriate selection (Ampicillin). Several colonies were picked from this LB agar plate after 12 hours and transferred into 2 mL LB medium with appropriate antibiotic (Ampicillin). The culture was grown for 12 hours at 220 rpm at 37 ^{o}C in a bacterial shaker. These cultures were subject to plasmid extraction using the GeneJet Plasmid miniprep kit. The eluted plasmids were then subject to EcoR1 restriction digest for 1 hour at 37 ^{o}C. The digested plasmids were run on a 0.7% agarose gel. The bacterial colony corresponding to the restriction digest which produced the predicted restriction fragments (Topo vector – 3.9 kb, insert – 3.1 kb for Strep/FLAG tagged FANCJ) was selected and expanded. The uncut plasmid from this positive colony was sent for sequencing by the Source Bioscience sequencing laboratory in Oxford.

Sequencing primers

Primer name	Sequence
M13F	5'-GTAAAACGACGGCCAG-3'
M13R	5'-CAGGAAACAGCTATGAC-3'

2.2.4 Analysis of Strep and FLAG tagged FANCJ protein

2.2.4.1 Whole cell extracts

All solutions and materials were kept on ice. To generate whole cell extracts, 1 x 10^8 cells were washed in phosphate buffered saline (PBS) and then resuspended in Urea buffer. The samples were sonicated at 10 amplitude microns for 15 seconds and protein quantified using BioRad protein assay.

2.2.4.2 Protein concentration determination by Bradford assay.

Protein concentrations were determined according to the method of Bradford (Bradford, 1976). On binding of Coomassie brilliant blue to proteins, the maximum absorption is shifted from 465 nm to 595 nm. The absorption at 595 nm is used to determine the protein concentration. Before the measurement of samples, a standard curve is prepared with BSA solution (conc. 1mg/mL):

200μL of 1x Bradford reagent (Blank control)
2μL BSA + 198μL 1 x Bradford reagent
4μL BSA + 196μL 1 x Bradford reagent
6μL BSA + 194μL 1 x Bradford reagent
8μL BSA + 192μL 1 x Bradford reagent
10μL BSA + 190μL 1 x Bradford reagent

198μL 1 x Bradford reagent was added to 2μL of the protein sample. Each sample was run in triplicate. Absorption was measured after 2 minutes at 595 nm. The standard curve allows the calculation of protein concentration in unknown samples.

2.2.4.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

Protein lysates containing 5X Laemmli buffer were denatured at 100^o C for 5 minutes and run on 7% acrylamide gels. Gels were electrophoresed in SDS PAGE running buffer at 25mA. Proteins were transferred to Hybond-C Extra Nitrocellulose membrane in transfer buffer for 10 hours at 400mA.

2.2.4.4 Western blotting

Nitrocellulose membranes were blocked in 5% (w/v) milk in TBST for 1 hour. Membranes were incubated with primary antibodies in 5% milk TBST for a period of 10 hours or more at 4^o C. The membranes were washed 5 times using TBST for 5 minutes each time, before incubating with the appropriate secondary antibody for 1-2 hours at room temperature. Membranes were washed as before and developed by addition of electrochemical luminescence reagent. Light produced by catalytic oxidation of luminol in the detection solution through horseradish peroxidase is used for detection. Membranes were exposed to Super RX Fuji Medical X-ray films and the films were developed.

2.2.4.5 Sensitivity assay

To test whether protein function had been compromised after knock-in of the tag and then unloxing, a cell viability assay was performed. Frozen aliquots of the cell lines, including wild type (negative control) and FANCJ-/-(positive control) were thawed out. After they had been growing for 4-5 days, 3000 cells/well were plated and incubated with increasing doses of cisplatin (50nM – 500nM). The cells were left to grow for 3 days and their cisplatin sensitivity was compared to the DT40 wild type and FANCJ-/- controls using Alamar Blue assay for viable cells. Alamar blue is an oxidation-reduction growth indicator. Innate metabolic activity maintains a reduced environment while inhibition of growth leads to oxidized environment. This difference in redox state produces change in colour of the indicator from blue (oxidized) to red (reduced). Data is collected using fluorescence. Fluorescence is monitored at 540 nm – excitation wavelength and 590 nm - emission wavelength.

2.2.4.6 Biochemical assays - Strep/FLAG Tandem affinity Purification (SF-TAP)

The SF-TAP tag (Figure 2.2.3) comprises a tandem Strep-tag II and a FLAG moiety optimized for rapid as well as efficient tandem affinity purification of native proteins and protein complexes in higher eukaryotic cells. Depending on the stringency of purification conditions, SF-TAP allows both the isolation of a single tagged-fusion protein of interest and purification of protein complexes under native conditions (Gloeckner et al 2009).

Figure 2.2.3. Cartoon representation of the C-terminal 2Strep/FLAG tag. (Gloeckner et al 2009) POI – protein of interest, C – C-terminal. Not to scale. Upper panel shows the relative positions of the two Strep and one FLAG tags. The two Strep tags are internal tags. The FLAG tag is the external C-terminal tag. Lower panel shows the amino acid sequences of the Strep and FLAG tags.

The principle of the tandem Strep/FLAG immunoprecipitation is that in the first step the furan ring of the histidine amino acid from the Strep tag mimics the furan structure of biotin and as such binds to streptactin beads, this is followed by washing the beads with wash buffer. In the second step, elution buffer saturated with desthiobiotin is used for elution of the SF-TAP fusion protein from the StrepTactin matrix. In the third step, the eluate from the Strep immunoprecipitation is loaded onto anti-FLAG M2 affinity matrix. The FLAG tag of the fusion protein binds to the anti-FLAG mouse monoclonal antibodies; this is followed by washing the matrix with wash buffer. Finally, in the fourth step, elution buffer saturated with FLAG octapeptide is used for elution of the SF-TAP fusion protein from the anti-FLAG M2 affinity matrix. The SF-TAP protocol represents an efficient, fast and straightforward purification of protein complexes (Gloeckner et al 2009). A cartoon description of the Strep/FLAG tandem affinity purification procedure is shown in Figure 2.2.4.

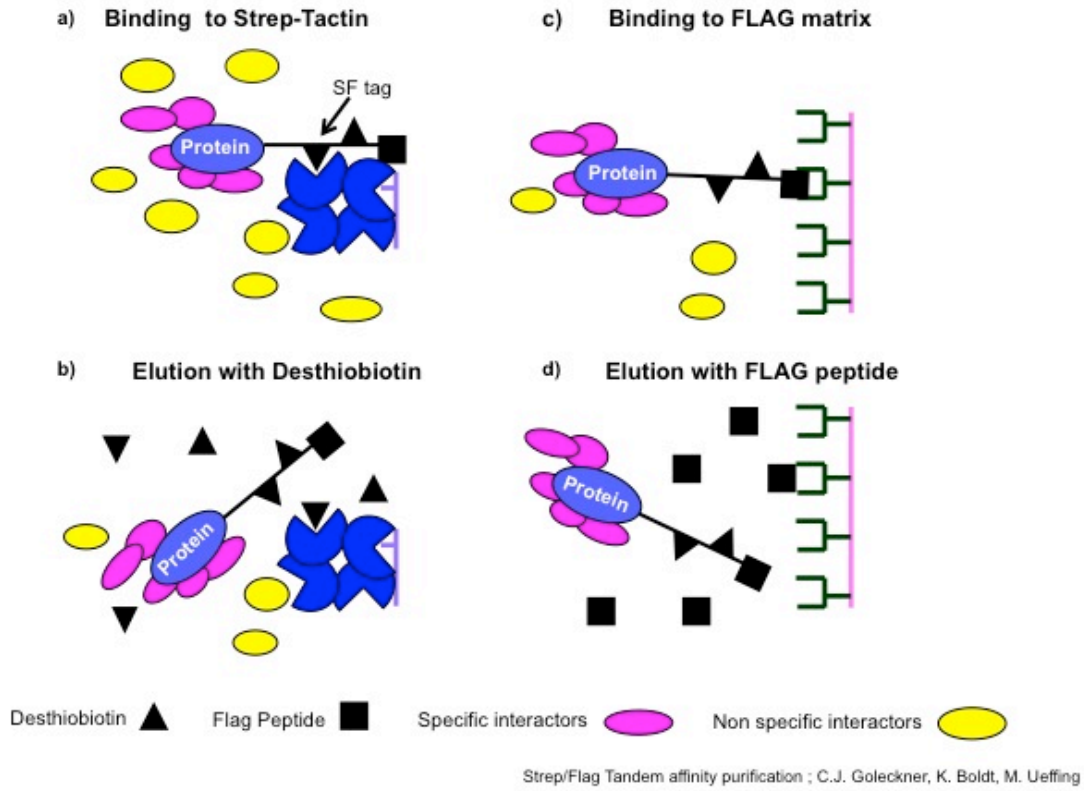


Figure 2.2.4. An overview of Strep/FLAG Tandem Affinity purification. Adapted from (Gloeckner et al 2009). The bait protein is denoted in purple colour.

The details of the Strep/FLAG immunoprecipitation are provided below.

2.2.4.7 Generation of cell lysates

To generate cell extracts, 1 x 10^8 cells were washed in ice cold phosphate buffered saline (PBS) and then incubated in lysis buffer for 1 hour at 4 ^oC. 25U/1000µL Benzonase was also added to the samples to digest genomic DNA and RNA. Samples were then centrifuged at 4 ^oC at 10,000 g for 15 min. Protein lysate supernatant was then transferred to clean microcentrifuge tube and protein quantified using BioRad protein assay.

2.2.4.8 Strep immunoprecipitation

StrepTactin resin was prepared by washing thrice with lysis buffer. Samples were incubated with 50µl Strep-Tactin resin for 2 hours at 4 ^oC for each purification. Samples were centrifuged at 400 g for 30 seconds at 4 ^oC and the supernatant was removed. Resin was washed 3 times using wash buffer. All centrifugation steps were carried out at 400 g for 30 seconds at 4 ^oC. StrepTactin resin was incubated with 500µL elution buffer for 4 hours at 4^oC. Supernatants were spun down at 400 g for 30 seconds at 4 ^oC to remove elution buffer. Fresh elution buffer was added for further incubation followed by two 15-minute elutions.

2.2.4.9 FLAG immunoprecipitation

The anti-FLAG M2 agarose resin was prepared by washing thrice with TBS. The eluate from the Strep immunoprecipitation was incubated with 50µl of FLAG agarose

resin for 6 hours at 4 ^{o}C. Samples were centrifuged at 400 g for 30 seconds at 4 ^{o}C and the supernatant removed. The resin was washed 3 times; once using wash buffer and twice using TBS. All centrifugation steps were carried out at 400 g for 30 seconds at 4 ^{o}C. The anti-FLAG M2 agarose resin was incubated with 500µL elution buffer for 4 hours at 4 ^{o}C. Supernatant was spun down at 400 g for 30 seconds at 4 ^{o}C to remove elution buffer. This was followed by 3.5-pH glycine elution for 20 minutes at 4 ^{o}C. The liquid eluate from FLAG peptide elution was then sent for analysis by Mass spectrometry.

2.2.4.10 Mass Spectrometry (MS)

MS was carried out in collaboration with Dr. Benedikt Kessler (Wellcome Trust Center, Oxford). Elution samples from the tandem Strep/FLAG immunoprecipitation were sent for in-solution digest followed by Electrospray Ionisation and Ion-trap MS in an attempt to identify bait protein and its interacting partners.

CHAPTER 3: RESULTS

3.1 GENERATION OF TAGGED CELL LINES

3.1.1 Introduction

To gain insights into the role of FANCJ in DNA repair, I generated a DT40 cell line expressing STREP/FLAG tagged FANCJ protein. To this end, I introduced in to the last exon of FANCJ an in-frame Strep/FLAG tag coding sequence. DT40 is an avian leucosis virus transformed chicken B-lymphocyte (Arakawa and Buerstedde 2004). It has a high level of conservation of the FA pathway between human and avian systems. Also, this cell line exhibits high ratio of targeted to random integration with transfected DNA constructs. These advantages made the DT40 cell line an ideal model to study the FA pathway. As such, this cell system was chosen for generating the Strep/FLAG tagged FANCJ cell line.

3.1.2 Targeted integration of Strep/FLAG tagged FANCJ constructs

Targeted integration of the FANCJ genomic loci was carried out using Strep/FLAG tagged FANCJ targeting constructs with two different drug resistance cassettes. The first allele of FANCJ was targeted using the Strep/FLAG tagged FANCJ targeting construct containing Neomycin resistance cassette. This was followed by antibiotic selection by Neomycin to select for positive colonies, which were then expanded and screened for targeted integration. The confirmed singly targeted Strep/FLAG tagged FANCJ clones (+/-) were expanded and subject to a second round of transfection with the Strep/FLAG tagged FANCJ targeting construct containing Blasticidin resistance cassette to target the second allele. This was then followed by antibiotic selection by Blasticidin to select for positive colonies, which were then expanded and screened for targeted integration. The confirmed doubly Strep/FLAG tagged FANCJ clone (+/+) was expanded and set up for transient transfection.

The schematic representation of steps involved in generating SF tagged FANCJ cell lines are shown in Figure 3.1.2. Panel A shows general structure of a linearized targeting vector. Panel B shows the structure of a linearized FANCJ targeting vector. Panel C is a cartoon representation of the wild type alleles of FANCJ present in the DT40 system. Panel D shows targeted integration of the FANCJ targeting construct containing Neomycin resistance cassette in the first allele. Panel E shows targeted integration of the FANCJ targeting construct containing Blasticidin resistance cassette in the second allele. Panel F shows desired stable targeted integration of the FANCJ targeting vectors in both the FANCJ alleles. This is followed by removal of the resistance cassettes (‘unloxing’). It is the unloxed cell line, which is used for further analysis.

3.1.3 PCR screening strategy to confirm targeted integration

To screen for stable targeted integration of the targeting constructs into the FANCJ genomic locus, a PCR-based screening system was developed. This system is based on the principle of a screening primer, annealing roughly 300 base pairs upstream of the 5' arm of the targeting construct and a primer annealing within the resistance cassette generating a band of predicted size. Generation of the band of calculated size, confirms positive targeted integration of the Strep/FLAG tagged FANCJ targeting construct into the FANCJ genomic locus.

This first stable targeted integration was identified by PCR screen using a primer pair with the forward primer annealing upstream of the 5′ arm, in the FANCJ genomic locus and a reverse primer annealing within the Neomycin resistance cassette (Figure 3.1.3A). For FANCJ, the heterozygous clones 7 and 9 were expanded and targeted for the second allele using the recycled targeting construct with a Blasticidin (BSR) resistance cassette. Identification of second stable targeted integration was confirmed by PCR screen using the forward primer annealing upstream of the 5′ arm, in the FANCJ genomic locus, as described before, and with a reverse primer annealing within the BSR resistance cassette (Figure 3.1.3B). The Strep/FLAG tagged FANCJ (Clone 3+/+) cell line was expanded and set up for transient transfection in order to remove the selection markers. This is an important step as it allows minimizing the modification introduced to the genomic locus of interest. This removal of selection markers is known as unloxing and the cell line with the integrated targeting constructs free of their resistance cassettes is said to be unloxed. Unloxing is done by transiently transfecting the cells with a plasmid containing the genomic sequence for Cre recombinase.

Cre recombinase belongs to the family of tyrosine recombinases derived from the P1 bacteriophage, which introduce cis-cleavages in DNA. (Sauer and Henderson 1988; Sternberg and Hamilton 1981). The enzyme uses a topoisomerase 1 like mechanism to carry out site specific recombination event between two 34 base pair DNA recognition sites known as loxP sites). LoxP recognition site consists of two 13 bp palindromic sequences, which flank an 8bp core sequence where recombination takes place. The DNA sites containing two loxP sequences can undergo fusion as the result of Cre mediated recombination, which ultimately leads to the removal of the DNA sequence between the two-loxP sites.

Unloxing was confirmed by loss of resistance to Neomycin and Blasticidin (data not shown). It is the unloxed homozygous cell line, which is used for further analysis.

3.1.4 Confirmation of Strep/FLAG tag integration into both the FANCJ alleles

To establish that both the alleles had the Strep/FLAG tag integrated in-frame with the genomic locus, a PCR screen was set up using a primer pair with the forward primer annealing 1900 base pairs upstream of the in-frame integrated Strep/FLAG tag and with the reverse primer annealing 1100 base pairs downstream of the in-frame integrated Strep/FLAG tag. An untargeted locus would generate a PCR product of calculated size whereas the tagged allele would generate a PCR product extended by the 150 base pairs of the Strep/FLAG tag (Figure 3.1.3C).

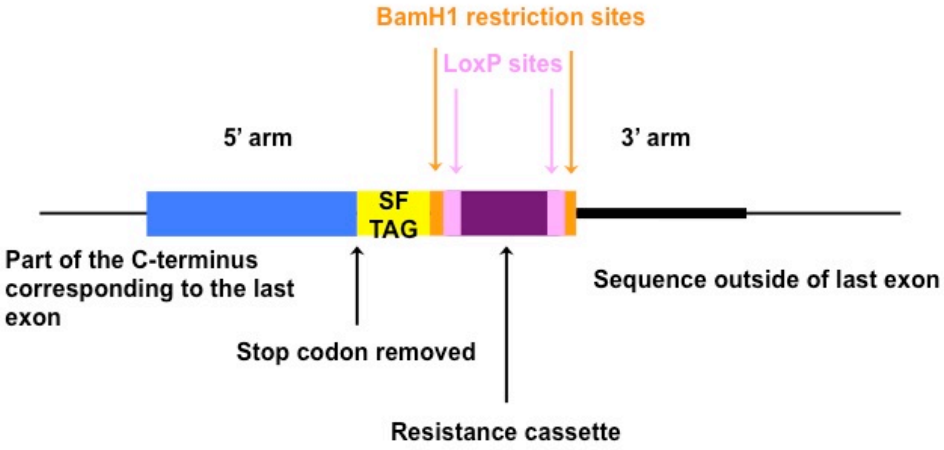
All the three PCR products appeared to run at approximately 3000 bps. Therefore, to confirm Strep/FLAG tag integration in the FANCJ cell lines, the three PCR products were subject to BamH1 and EcoR1 restriction double digest. Strep/FLAG tag integration introduces a BamH1 and EcoR1 site and as such, only the PCR products from FANCJ Strep/FLAG tagged cell lines would generate restriction fragments of calculated size, proving targeted integration of the Strep/FLAG tag into the FANCJ loci. The PCR product from the wild type (WT – untargeted locus) would remain unaffected. This is exactly what was observed after BamH1 and EcoR1 restriction double digest of the three PCR products (Figure 3.1.3D).

3.1.5 Confirmation of in-frame Strep/FLAG tag integration into the FANCJ genomic locus

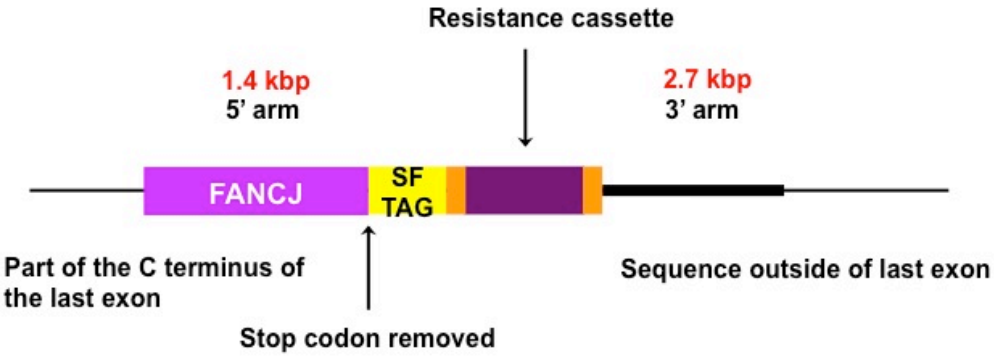
The uncut PCR product from Strep/FLAG tagged FANCJ clone 3.1 was cloned into Topo TA cloning vector. This uncut cloned plasmid was sent for sequencing using the provided sequencing primers to confirm in-frame Strep/FLAG tag integration into the FANCJ genomic locus.

The sequencing results confirmed the in-frame targeted integration of the two Strep and one FLAG tags into the FANCJ genomic loci (Figure 3.1.5A and 3.1.5B)

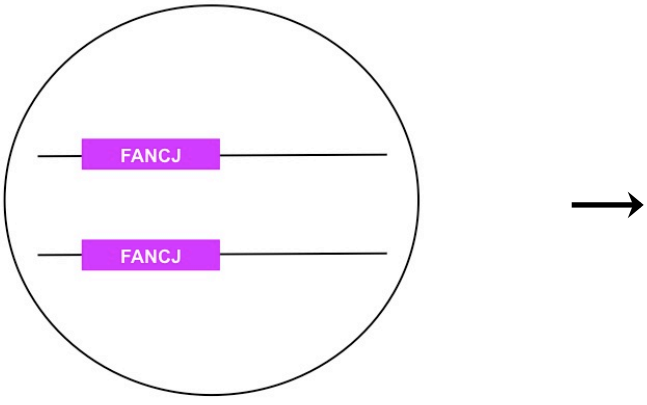
A


BamH1 restriction sites
LoxP sites
5' arm
3' arm
SF
TAG
Part of the C-terminus corresponding to the last exon
Stop codon removed
Sequence outside of last exon
Resistance cassette

B


Resistance cassette
1.4 kbp
5' arm
2.7 kbp
3' arm
FANCJ
SF
TAG
Part of the C terminus of the last exon
Stop codon removed
Sequence outside of last exon

C


FANCJ
FANCJ

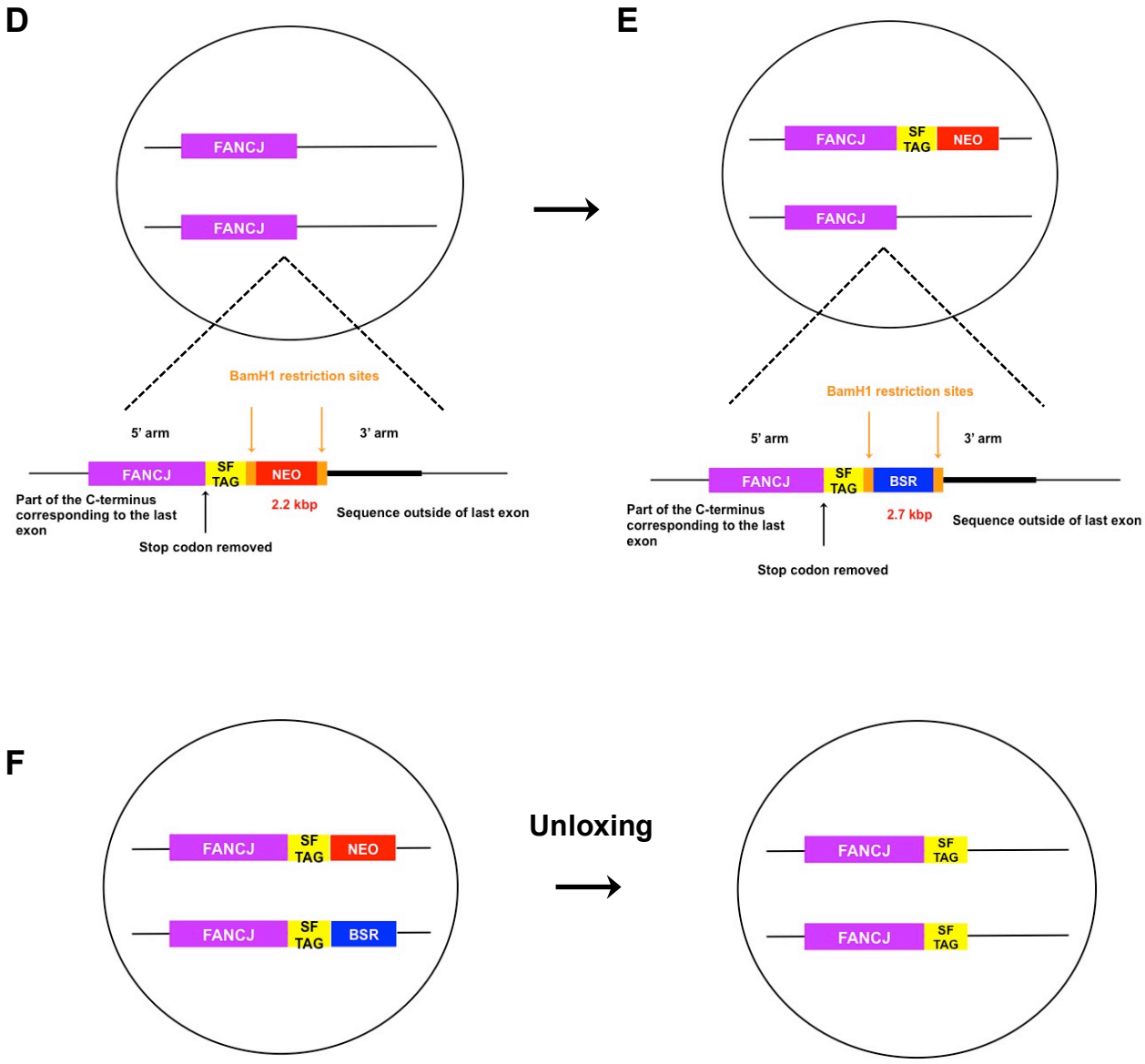


Figure 3.1.2. Strategy to generate SF-tagged FANCJ cell line.
Schematic representation of the targeting vectors and the steps involved.

(A) A Strep/FLAG knock-in targeting vector. Not to scale and shown in linear form. BamH1 sites are used to recycle the resistance cassettes, this allows for reuse of the same targeting vector. The LoxP sites are used to remove the resistance cassette once targeted integration is confirmed via PCR screen.

(B) SF-tagged FANCJ targeting vector. Not to scale and shown in linear from. The lengths of the 5' arm and 3' arm are provided.

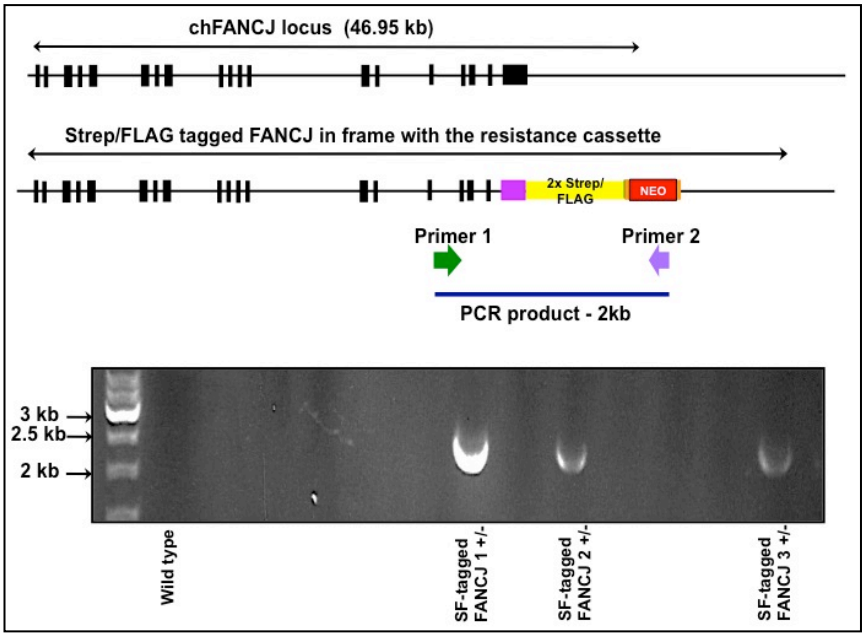
(C) Untagged wild-type FANCJ genomic loci in DT40 cell (shown in panel B).

(D) Stable targeted integration of the SF-tagged FANCJ targeting vector (shown in panel B) containing a Neomycin (Neo) resistance cassette into the first FANCJ allele.

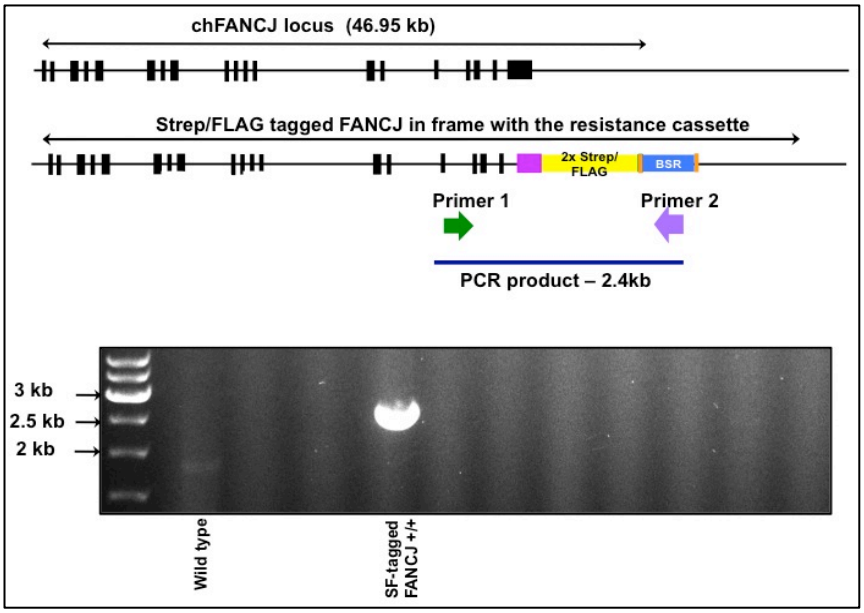
(E) Stable targeted integration of the SF-tagged FANCJ targeting vector (shown in panel B) containing a Blasticidin (Bsr) resistance cassette, into the second FANCJ allele.

(F) SF-tagged FANCJ cell line with desired targeted integration of both the targeting vectors. This cell line is 'unloxed' to remove both the resistance cassettes in order to minimize modification to the FANCJ genomic locus. It is this cell line that is used to further analysis (shown in panel C).

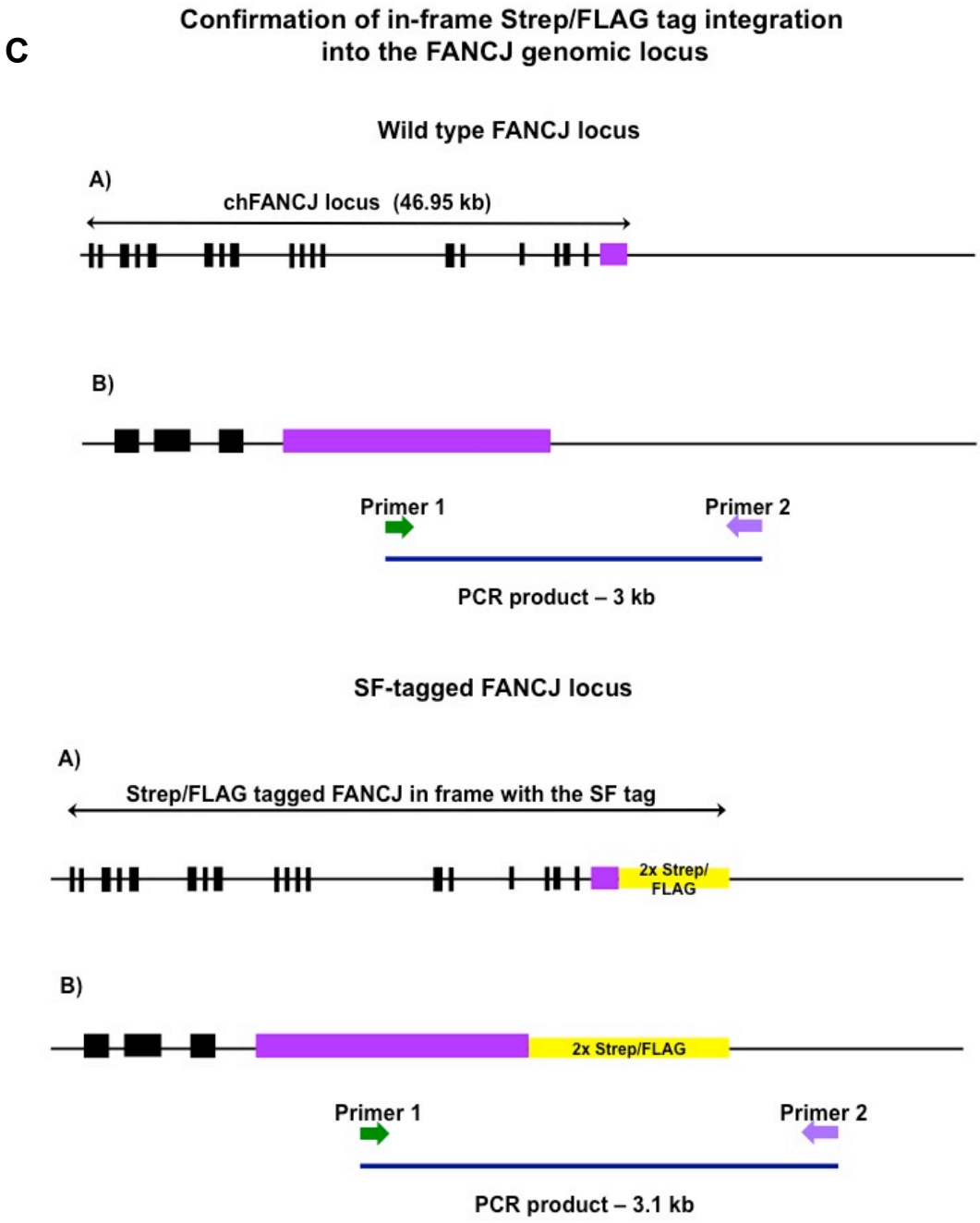
A


chFANCJ locus (46.95 kb)
Strep/FLAG tagged FANCJ in frame with the resistance cassette
2x Strep/ FLAG
NEO
Primer 1
Primer 2
PCR product - 2kb
3 kb
2.5 kb
2 kb
Wild type
SF-tagged FANCJ 1 +/-
SF-tagged FANCJ 2 +/-
SF-tagged FANCJ 3 +/-

B

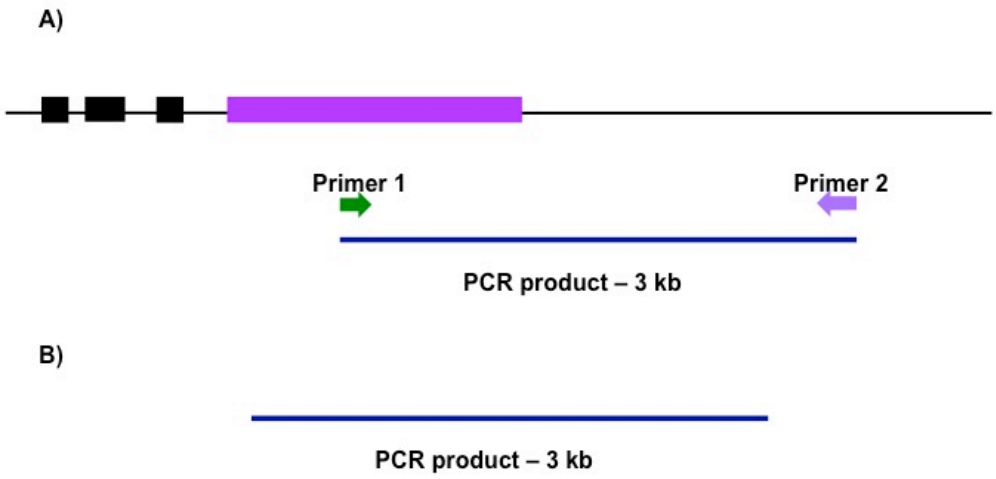

chFANCJ locus (46.95 kb)
Strep/FLAG tagged FANCJ in frame with the resistance cassette
2x Strep/ FLAG
BSR
Primer 1
Primer 2
PCR product – 2.4kb
3 kb
2.5 kb
2 kb
Wild type
SF-tagged FANCJ +/+

C

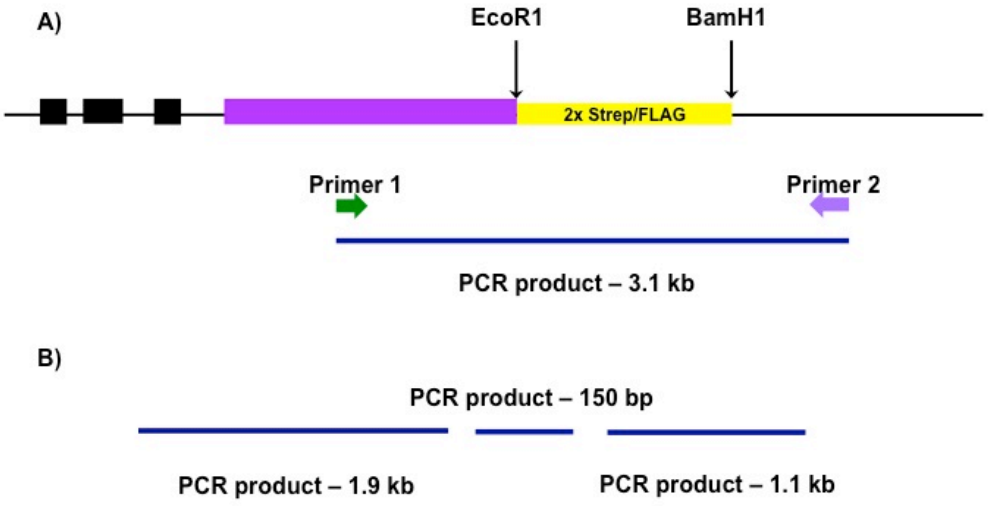

Confirmation of in-frame Strep/FLAG tag integration into the FANCJ genomic locus
Wild type FANCJ locus
A)
chFANCJ locus (46.95 kb)
B)
Primer 1
Primer 2
PCR product – 3 kb
SF-tagged FANCJ locus
A)
Strep/FLAG tagged FANCJ in frame with the SF tag
2x Strep/ FLAG
B)
2x Strep/FLAG
Primer 1
Primer 2
PCR product – 3.1 kb

D

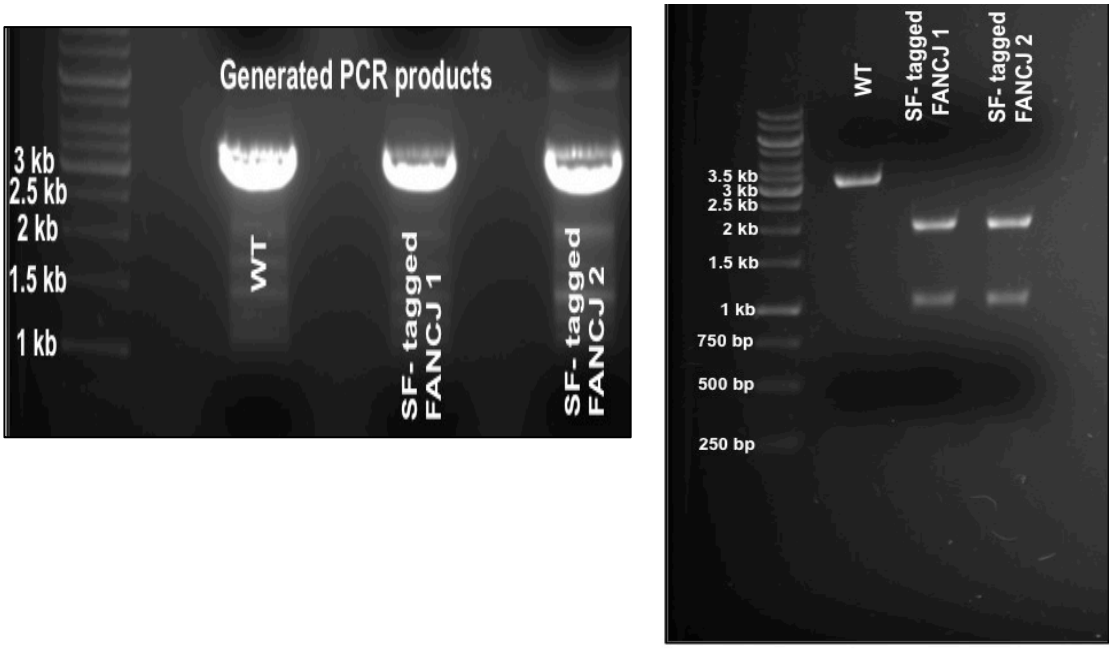
EcoR1 & BamH1 double digestion of Wild type PCR product



EcoR1 & BamH1 double digestion of SF tagged FANCJ PCR product



E



Restriction digestion using EcoR1 & BamH1

Figure 3.1.3. PCR screen to establish stable targeted integration of both the targeting vectors into the FANCJ genomic loci.

(A) Schematic representation of untagged DT40 FANCJ locus and SF-tagged FANCJ locus with the Neomycin resistance cassette present. A primer pair consisting of one primer annealing upstream of the 5' arm of the targeting vector, and a second primer annealing within the Neomycin resistance cassette will produce a PCR product of 2 kbp (kilo base pairs) only when stable targeted integration has been achieved. The run PCR products confirmed stable targeted integration of the SF-tagged FANCJ targeting vector with the Neomycin resistance cassette in the first allele of the FANCJ genomic locus in three clones.

(B) Schematic representation of untagged DT40 FANCJ locus and SF-tagged FANCJ locus with the Blasticidin resistance cassette present. A primer pair consisting of one primer annealing upstream of the 5' arm of the targeting vector, and a second primer annealing within the Blasticidin resistance cassette will produce a PCR product of 2.4 kbp only when stable targeted integration has been achieved. The run PCR products confirmed stable targeted integration of the SF-tagged FANCJ targeting vector with the Blasticidin resistance cassette in the second allele of the FANCJ genomic locus of one clone.

(C) Strategy to establish in-frame SF-tagging of both the FANCJ alleles. A primer pair located 1900 base pairs (bp) upstream and 1100 bp downstream of the Strep/FLAG tag will produce a PCR product of calculated size in the untagged FANCJ locus. The same primer pair will not produce a PCR product if the FANCJ allele has both the resistance cassettes present. In-frame Strep/FLAG-tagged FANCJ free of resistance cassettes will generate a PCR product extended by 150 bp of the Strep/FLAG-tag. Not to scale.

(D) Schematic representation of the EcoR1 and BamH1 double digestion to confirm stable targeted integration of both targeting vectors into the FANCJ genomic locus. The sizes of the restriction digestion products are given. Not to scale.

(E) The run PCR products confirmed in-frame SF-tag integration in the FANCJ cell lines. WT refers to wild type FANCJ, and Strep/FLAG-tagged FANCJ 1 and 2 refer to the doubly tagged Strep/FLAG-tagged FANCJ cell lines with both the resistance cassettes removed. EcoR1 and BamH1 double restriction digest of the PCR products from the three cell lines produces fragments of calculate size (1.9 kb and 1.1 kb) only in the Strep/FLAG-tagged FANCJ cell lines, as seen in the gel. The 150 bp restriction product is not seen, possibly due to the small size of the product.

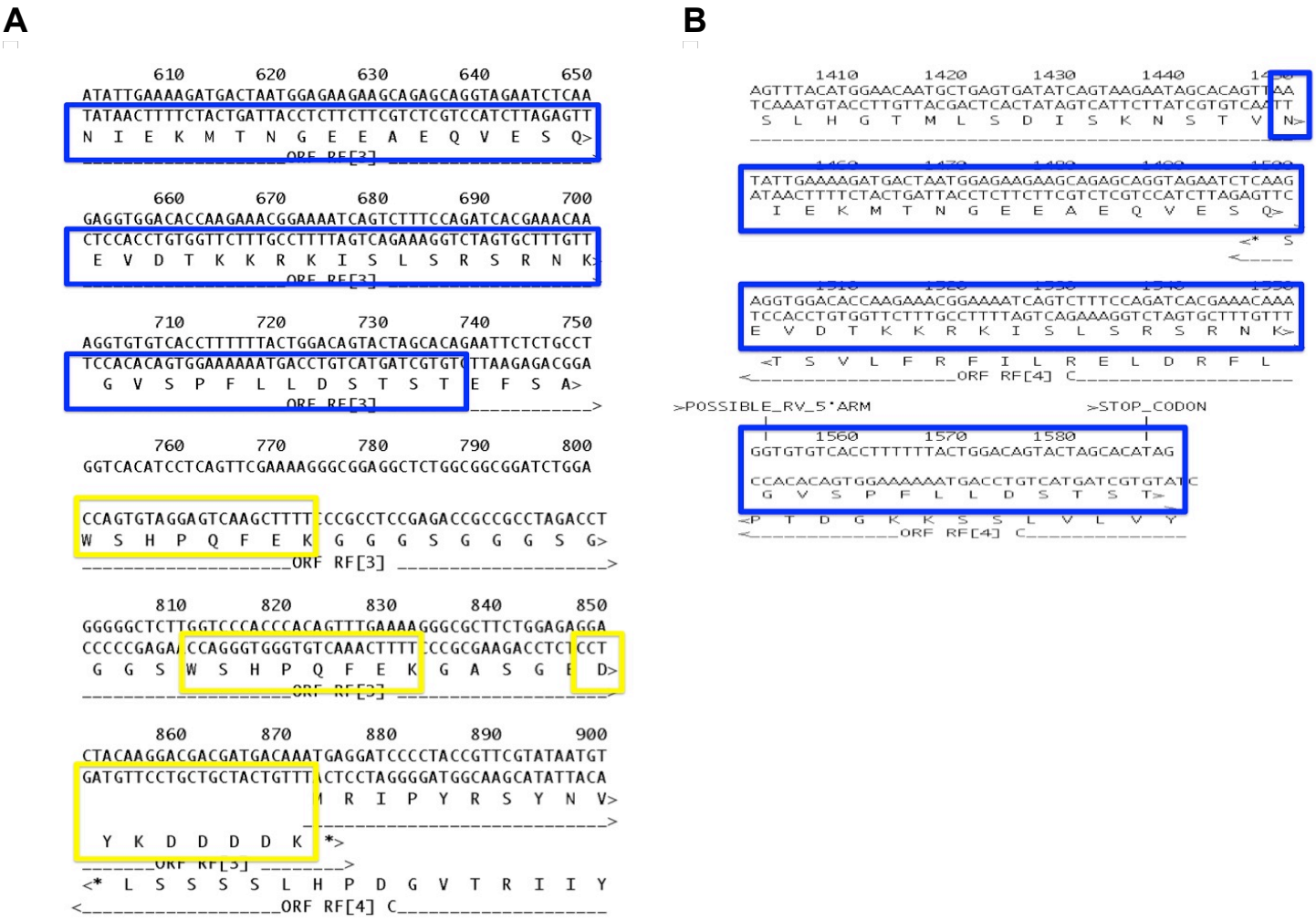


Figure 3.1.5. DNA sequencing results from Strep/FLAG tagged FANCJ cell line and the Wild Type FANCJ cell line to confirm in-frame tag integration into the FANCJ genomic loci.

(A) Part of the C-terminus DNA sequence from the Strep/FLAG tagged FANCJ cell line. The blue boxes indicate the sequences which are present both in the Strep/FLAG tagged FANCJ cell line and the Wild Type FANCJ. The yellow boxes show the in-frame two Strep and one FLAG tags.

(B) Part of the C-terminus DNA sequence from the Wild Type FANCJ cell line. Blue boxes indicate the sequences which are present both in the Strep/FLAG tagged FANCJ cell line and the Wild Type FANCJ.

3.2 RECOMBINANT FANCJ PROTEIN ANALYSIS

3.2.1 Introduction

The identified homozygously Strep/FLAG tagged FANCJ cell lines were expanded and subject to protein analysis to confirm the expression of recombinant Strep/FLAG tagged FANCJ protein, the functionality of the recombinant protein and whether it was folding properly so that both the Strep/FLAG tags were available for binding.

3.2.2 Confirmation of the recombinant Strep/FLAG tagged FANCJ protein expression

To confirm the expression of the recombinant Strep/FLAG tagged FANCJ protein, the unloxed Strep/FLAG tagged FANCJ cell lines were expanded and whole cell lysates derived from them were subject to an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting (Figure 3.2A). The membrane was incubated with anti-Strep antibody to check for expression of the recombinant Strep/FLAG tagged FANCJ protein. This appearance of a band of predicted size, i.e. 140kDa (Figure 3.2A) confirmed that the recombinant protein was being expressed in the heterozygous and homozygous Strep/FLAG tagged FANCJ-expressing cell lines.

3.2.3 Availability of Strep tag(s)

To confirm the availability of the Strep tag(s) for immunoprecipitation, a Strep only immunoprecipitation was performed. This was successful and confirmed the accessibility of the Strep tag(s). This indicated that the internal Strep tag(s) were available for binding, and later experiments confirmed the availability of the FLAG tag for binding (Figure 3.3.3.3.B and 3.3.3.3.C). The presence of TOPBP1, a known interacting partner in the Strep immunoprecipitation indicated that the presence of the tag had not interfered with this best characterized C-terminal interaction of FANCJ, and therefore, it was likely that the introduction of the Strep/FLAG tags had not interfered with any other interactions of FANCJ (Figure 3.2B).

3.2.4 Cell viability assay

To confirm that the endogenous introduction of the Strep/FLAG tags had not interfered with the protein function, the Strep/FLAG tagged FANCJ was subject to a cell viability assay. To ascertain whether the expressed Strep/FLAG tagged FANCJ protein is functional, Strep/FLAG tagged FANCJ expressing cells were tested for sensitivity to the DNA cross-linking agent cisplatin (CP) that causes replication-fork stalling and DSBs in S-phase (Figure 3.2C). The stock solution of CP was stored in DMSO, which is known to decrease the effectiveness of the drug, as such, we observed reduced killing among the various cell lines at the given concentrations. The Strep/FLAG tagged FANCJ cell lines were no more sensitive to CP than the wild type control whereas FANCJ -/- cells were extremely sensitive to this drug. This indicated that tagging the protein had not markedly interfered with its cellular functions.

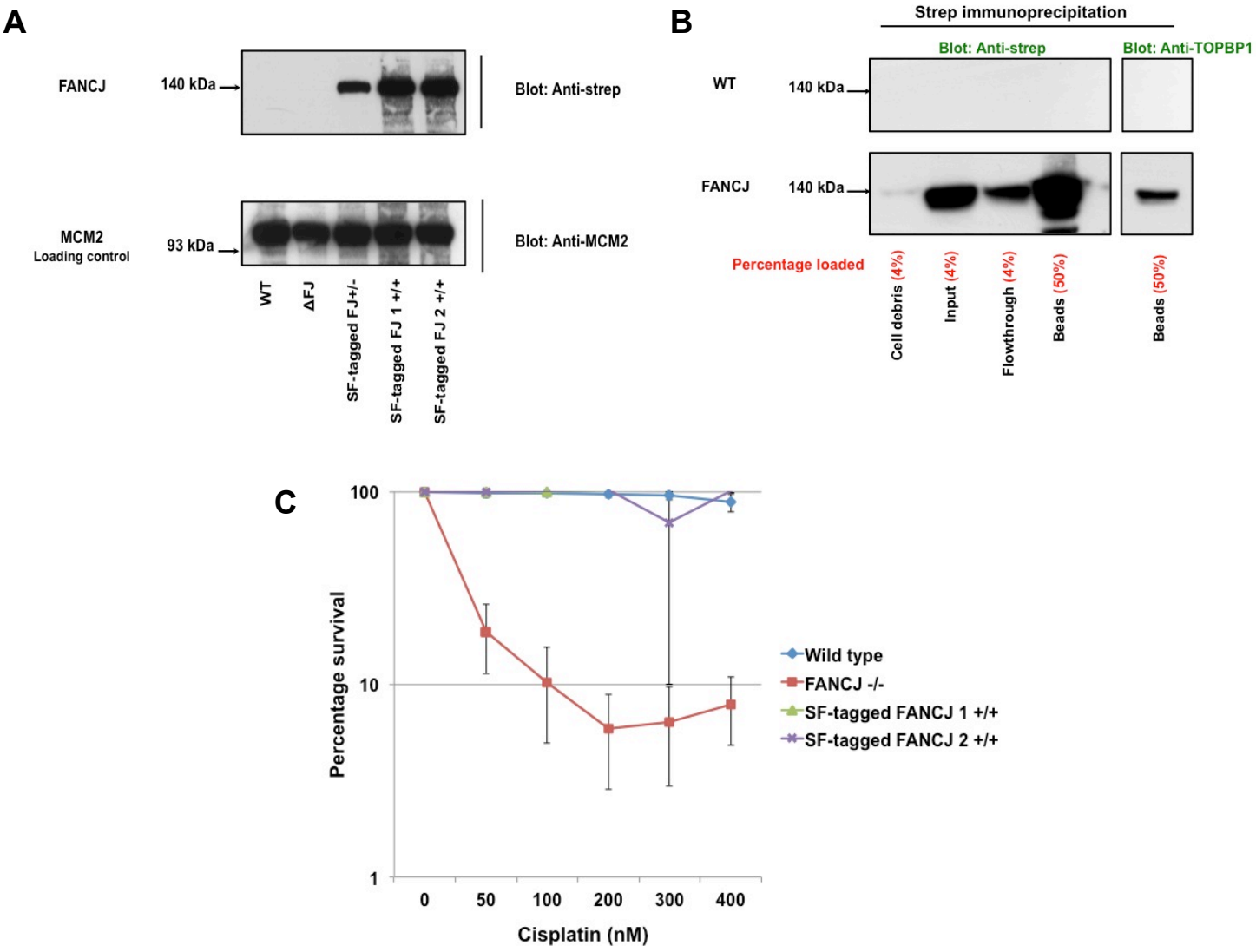


Figure 3.2. Characterization of Strep/FLAG tagged FANCJ cell lines.

(A) Strep/FLAG-tagged FANCJ expression was confirmed in the generated cell lines. WT refers to whole cell lysate from wild-type or untargeted FANCJ cell line, ΔFJ refers to whole cell lysate from FANCJ knock out cell line, FJ +/- refers to whole cell lysate from parental cell line with only one allele Strep/FLAG tagged, and Strep/FLAG tagged FANCJ 1 and 2 refer to the whole cell lysates from the unloxed, doubly Strep/FLAG-tagged FANCJ cell lines 1 and 2. Loading control MCM2 is also shown. FANCJ – 140 kDa, MCM2 – 110 kDa.

(B) Strep immunoprecipitation of Strep/FLAG-tagged FANCJ confirming availability of the two Strep tags. The presence of a known interacting partner of FANCJ, TOPBP1 in the immunoprecipitation indicates that the c-terminal Strep/FLAG-tag has not interfered with this key interaction of FANCJ. FANCJ – 140 kDa, TOPBP1 – 170 kDa. TOPBP1 seems to be running at 140 kDa because the gel was run for a shorter period of time (1 hour), which gave forth the observed low resolution between the two different proteins.

(C) Cell survival assay to generate sensitivity profile for unloxed Strep/FLAG-tagged FANCJ 1 and 2 cell lines with wild-type and FANCJ-/- cell lines used as controls. Strep/FLAG-tagged FANCJ 1 and 2 refer to the Strep/FLAG-tagged FANCJ cell lines with both the alleles tagged and the resistance cassettes removed. Cells were exposed to increasing doses of the DNA crosslinking agent Cisplatin and left for 3 days. The sensitivity profile of the Strep/FLAG-tagged cell lines is similar to wild type indicating that the tag has not markedly interfered with protein function. Standard errors are shown on graphs. Note that the curve from SF-tagged FANCJ 1 +/+ is being superimposed by the curves from SF-tagged FANCJ 2 +/+ and Wild type.

3.3 SF-TAP (STREP/FLAG TANDEM AFFINITY PURIFICATION) OPTIMIZATION

3.3.1 Introduction

Tandem affinity purification (TAP) is a tool for studying protein-protein interactions. This technique is used to identify and characterize protein complexes. It involves creating a recombinant protein where the protein has a TAP tag on either its N-terminus or its C-terminus. This recombinant protein is recovered from the cells and subject to tandem affinity purification, which effectively and specifically purifies the recombinant protein along with its interacting partners. The affinity purified recombinant protein and its interacting protein(s) are then analyzed by SDS-PAGE and/or identified by Mass Spectrometry (MS).

3.3.2 The Strep/FLAG-TAP protocol

The Strep/FLAG-TAP protocol was adapted from Glockner et al 2009. Here, cells are lysed under physiological conditions; this should preserve all the major interactions of the bait protein, followed by incubation with Streptactin matrix and anti-FLAG M2 agarose resin to obtain purified bait protein and its interacting partners.

The Strep immunoprecipitation protocol was being optimized in the laboratory at the time when these experiments were performed. Thus, I began by optimizing the FLAG immunoprecipitation. In order to establish the incubation time which allowed for maximum recovery of bait protein, I incubated cleared whole cell lysate from SF-tagged FANCI cell line (The SF-tagged FANCI cell line had been previously generated and characterized in the laboratory and as such, it was used as a positive control to confirm that that immunoprecipitations were working) with anti-FLAG M2 agarose resin for 1, 3, 6 hours and overnight (12 hours) (Figure 3.3.3.1B). I wanted to achieve a balance between the time required for the pulldown and the maximum recovery of the bait protein and its interacting partners. Increase in time during incubation might lead to dissociation of the possible DNA repair complexes formed. As such, I chose the 6-hour incubation, as it clearly allowed for increased recovery of the bait protein.

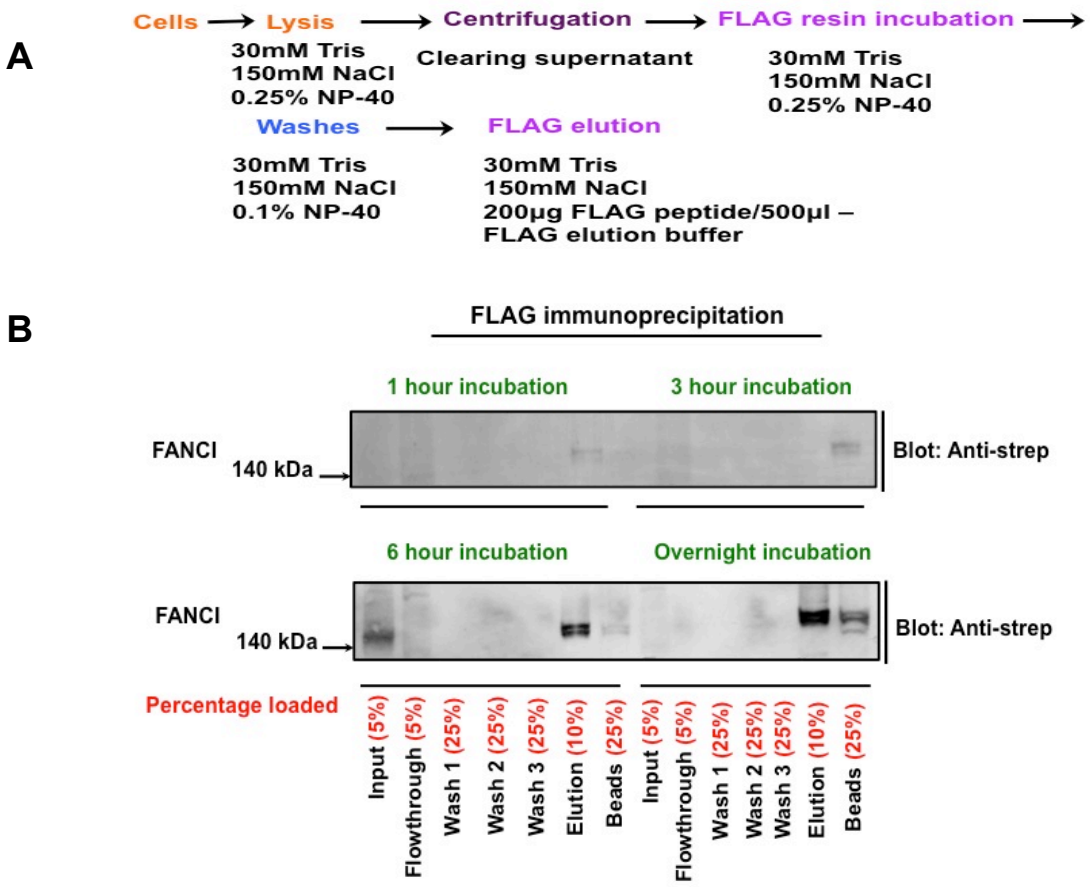


Figure 3.3.3.1. Incubation time for FLAG immunoprecipitation.

(A) Flowchart of FLAG immunoprecipitation.

(B) To establish the incubation time for FLAG immunoprecipitations, which allow for maximum recovery of bait protein (Strep/FLAG-tagged FANCI). FLAG immunoprecipitations for 1, 3, 6 hour and overnight was performed. It clearly showed that 6-hour incubation allows for the maximum recovery of bait protein. The loading control (input) was not clearly visualized, possibly due to unequal antibody incubation.

This was followed by two independent Strep immunoprecipitation procedures (Figure 3.3.3.2B and Figure 3.3.3.2C) and two independent Strep/FLAG tandem affinity immunoprecipitations (Figure 3.3.3.3B and 3.3.3.3C) on SF-tagged FANCJ along with DT40 wild-type as negative control, where SF-tagged FANCI was again used as a positive control. All the independent experiments confirmed successful and reproducible immunoprecipitation.

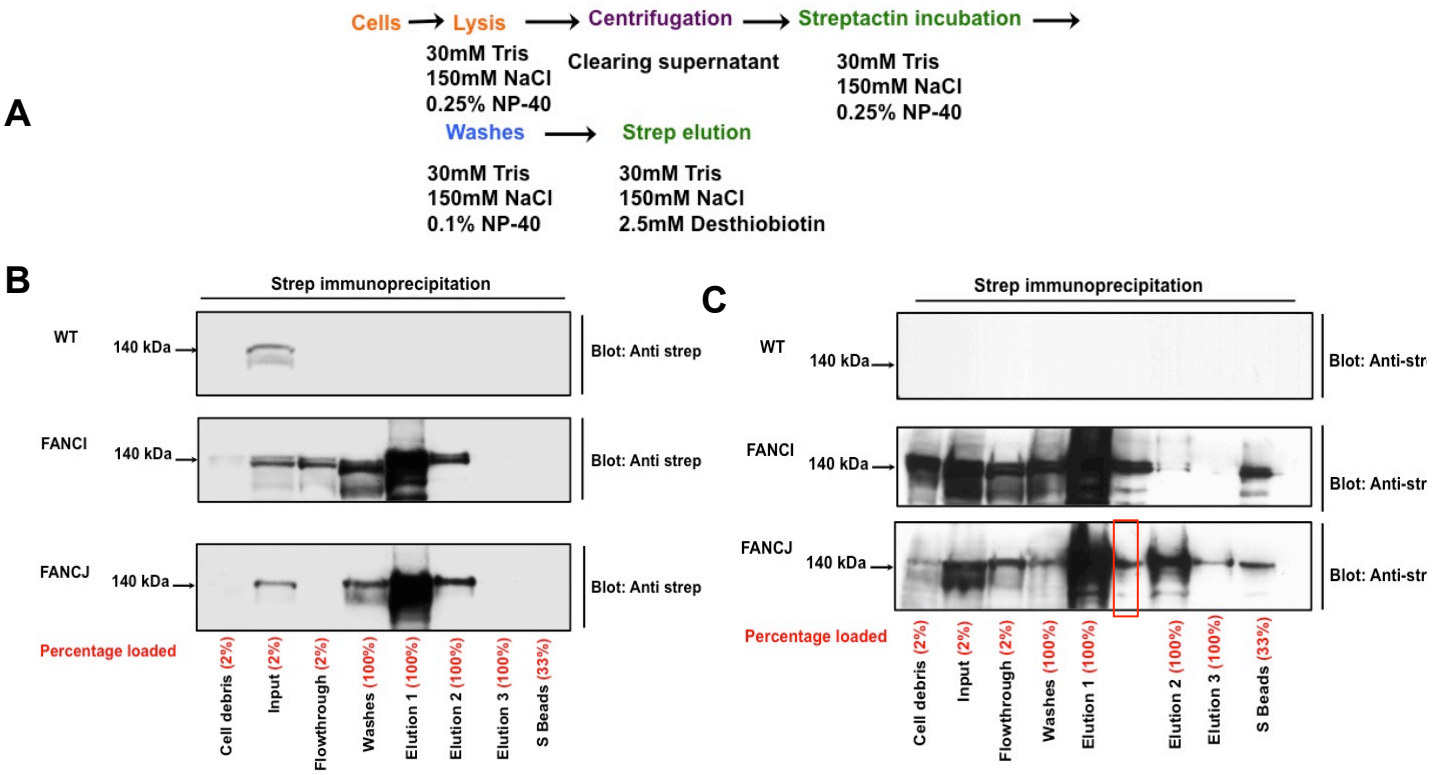


Figure 3.3.3.2. Strep immunoprecipitations.

(A) Flowchart of Strep immunoprecipitation.

(B) and (C) To establish reproducible Strep immunoprecipitations of SF-tagged FANCJ, two independent Strep immunoprecipitations were carried out using WT cells as negative control - WT refers to wild type parental cell line DT40 – and FANCI as positive control – FANCI refers to Strep/FLAG-tagged FANCI. Presence of a band in the WT western blot (Panel B) indicates contamination of samples. The red box (Panel C) indicates an unloaded well.

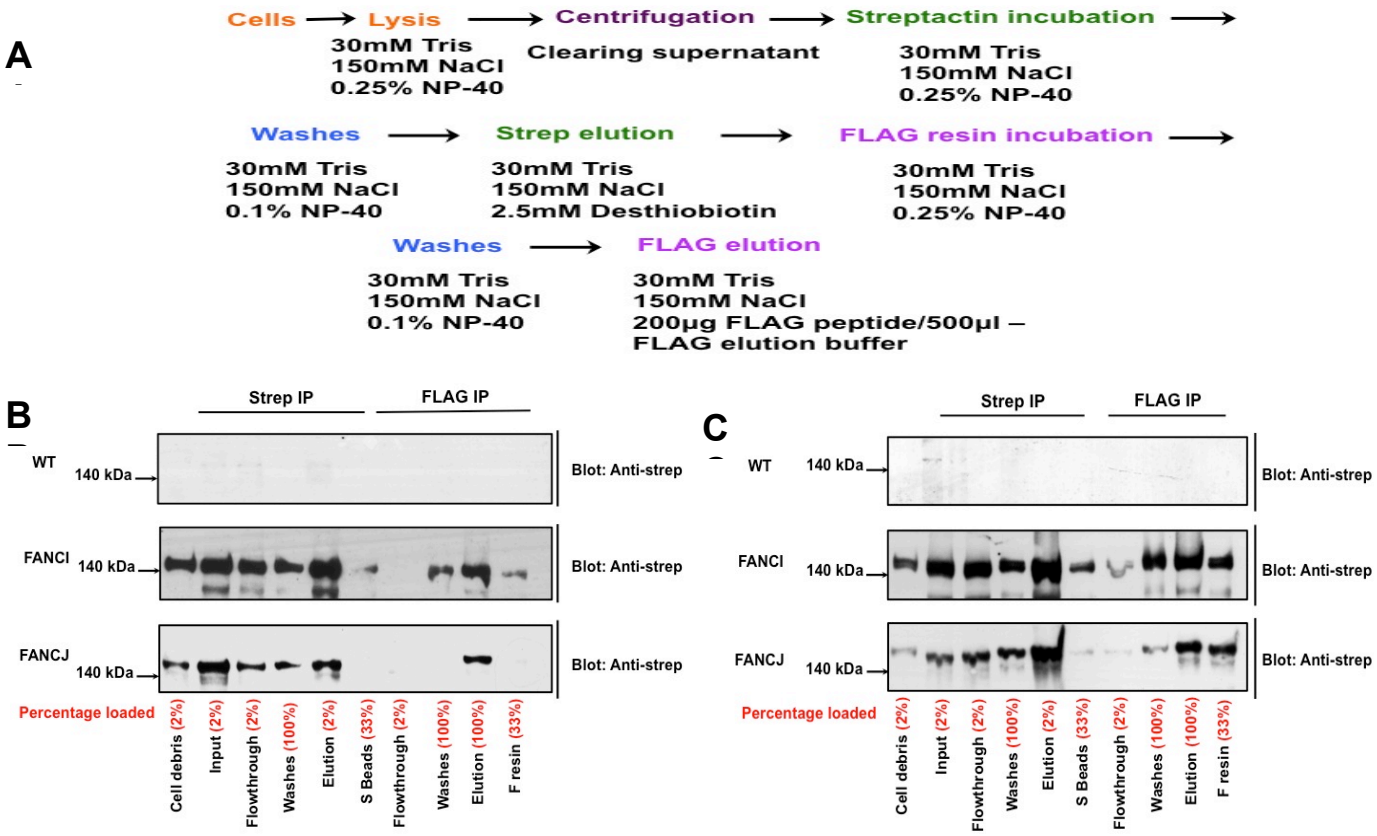


Figure 3.3.3.3. Strep/FLAG immunoprecipitations.

(A) Flowchart of Strep/FLAG immunoprecipitation.

(B) and (C) To establish reproducible Strep/FLAG tandem affinity immunoprecipitations of SF-tagged FANCJ, two independent Strep/FLAG tandem affinity immunoprecipitations were carried out using WT cell line as negative control - WT refers to wild type parental cell line DT40 – and FANCI as positive control – FANCI refers to Strep/FLAG-tagged FANCI.

Another independent SF-TAP (Figure 3.3.3.4) with Strep/FLAG tagged FANCJ cell lines and DT40 wild-type as negative control was performed and eluted samples sent for analysis by Mass Spectrometry (MS).

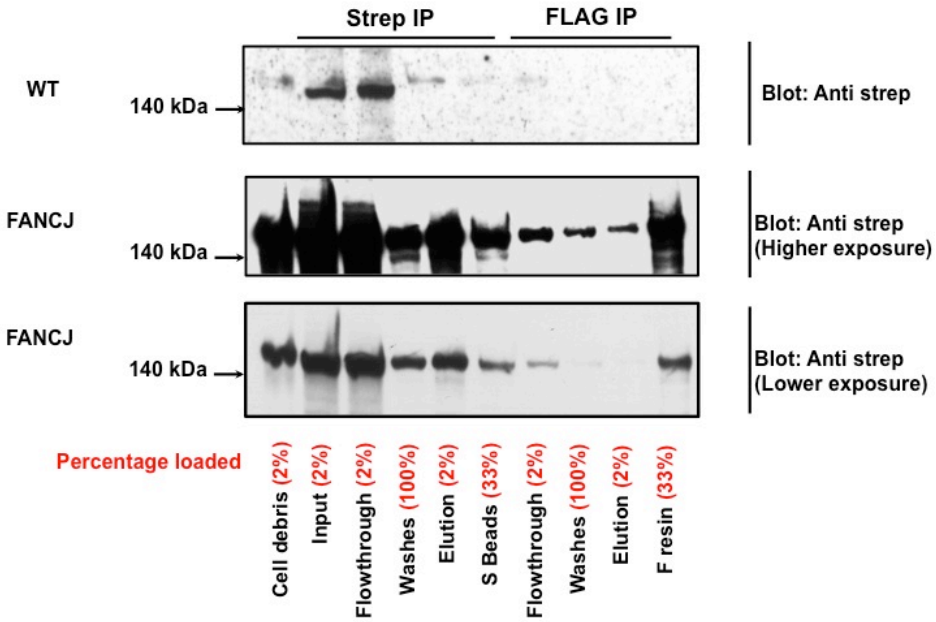


Figure 3.3.3.4. Scaled-up SF-TAP Immunoprecipitation.
Upper panel shows immunoprecipitation from DT40 wild type as control and lower panel shows immunoprecipitation from Strep/FLAG-tagged FANCJ sent for MS with two exposures. Presence of SF-tagged protein in the wild type indicates cross contamination. The S beads denote the boiled Streptactin matrix. The F resin denotes the boiled anti-FLAG M2 agarose resin. The presence of bands in the wild type indicates contamination of samples.

Cultures were scaled up to 2 litres with cell density being 0.5 x 10^6 per mL. The final FLAG eluate from Strep/FLAG tagged FANCJ and wild type was sent to MS for an in-solution digest with Trypsin to further analyze the identity of the proteins that associate or complex with the SF-tagged FANCJ. MS was carried out in collaboration with Dr. Benedikt Kessler (Wellcome Trust Centre, Oxford). Unfortunately, the bait protein was not detected. Even though tagged FANCJ could be clearly seen on the western blot (Figure 3.3.3.4), MS was not able to detect it. MS is able to detect proteins in the range of femtograms, whereas an immunoblot can detect proteins in the picogram range. Hence, it was concluded that the yield of protein(s) from the SF-TAP was somehow insufficient for MS. Therefore; the SF-TAP protocol was modified by a change in strategy to detect the bait protein and its interacting partners.

Another scaled-up Strep only immunoprecipitation on the SF-tagged FANCJ cell line with DT40 wild-type as control was performed (Figure 3.3.3.5).

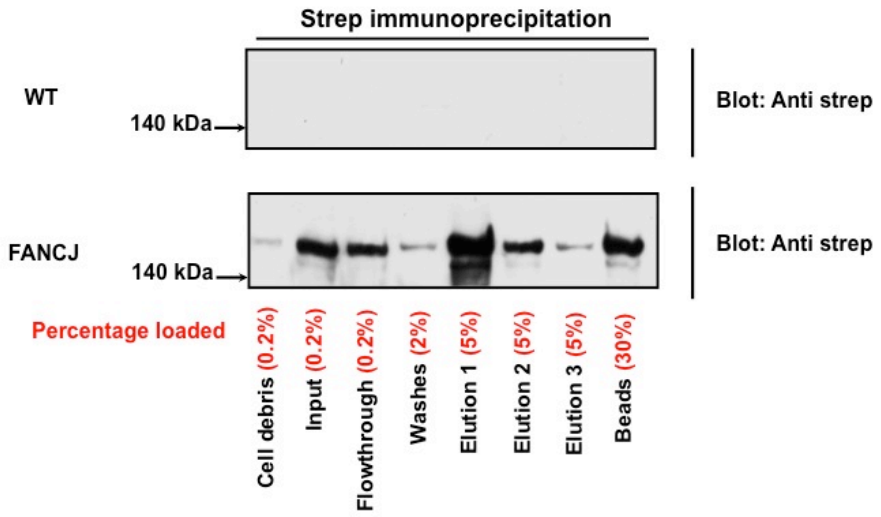


Figure 3.3.3.5. Scaled-up Strep immunoprecipitation.
Upper panel shows immunoprecipitation from DT40 wild type as control and lower panel shows immunoprecipitation from SF-tagged FANCJ sent for MS. The Beads denote the boiled Streptactin matrix. MS was able to identify SF-tagged FANCJ in Elution 1.

Elution 1 was sent for MS analysis, which was able to confirm the presence of FANCJ (Table 3.3.3.1 and Table 3.3.3.2). The FANCJ peptides detected by MS have been shown in Figure 3.3.3.6 and the FANCJ peptides generated by the tryptic digest of FANCJ proteins are shown in Figure 3.3.3.7. The sequence coverage, i.e. the total number of FANCJ peptides identified in percentage was 39%. Also, unfortunately, no published interacting partners were identified. This indicates the need to further optimize the immunoprecipitation (see discussion and future work).

3.3.3 Mass Spectrometry

Mass spectrometry is an analytical technique, which is used to measure the mass to charge ratio of charged particles. In proteomics, it is used to identify proteins by determining the mass of its peptides and by determining the amino acid sequence of these peptides. MS works on the principle of ionizing peptides to generate charged particles and then measuring their mass to charge ratios.

In order to identify the interacting partners of FANCJ, we used Ion-trap mass spectrometer to analyze the complex peptide mixture (Strep/FLAG or Strep only eluate) with Electrospray Ionization (ESI) as the ion source. Here, the eluted TAP sample was subject to a tryptic digest. This sample was then subject to a LC MS/MS (liquid chromatography followed by Mass Spectrometry followed by Mass spectrometry). In the first step, i.e. LC, Reverse Phase Chromatography was used to separate the generated peptides based on their hydrophobicity. Then, these peptides where subject to the first round of MS where, they were ionized and their mass to charge ratio analyzed. Once this ratio was analyzed, these peptides were then subject to another round of MS, where Collision Induced Dissociation (CID) was used to determine their amino acid sequence. The generated MS/MS spectra were analyzed and the identification of peptides was done against a decoy chicken database with the help of the MASCOT (Matrix Science) search engine.

MS was able to detect the bait protein the in Strep/FLAG tagged FANCJ elution sample (Table 3.3.3.1). The bait protein was not detected in the wild type control (Table 3.3.3.2). Unfortunately, no known FANCJ interacting proteins were detected in the Strep/FLAG tagged FANCJ elution sample. This undermines the validity of the immunoprecipitation and suggests that the interacting partners are being lost during one of the steps involved in the immunoprecipitation and as such, the immunoprecipitation protocol to needs to be optimized further.

In tables 3.3.3.1 and 3.3.3.2, S.No. or serial number indicates ordering on basis of the abundance of a protein in the elution sample, as detected by MS. Total number of peptides indicates the total number of peptide sequences for a given protein, detected in the elution sample. Unique peptides indicate the total number of unique peptide sequences of a given protein identified in the elution sample.

FANCJ MS Peptide profile

S.No.	Name of Protein	Total number of peptides	Unique peptides
1	Biotinyl peptide fragment	6980	132
2	Actin, cytoplasmic type 5	1929	27
3	Heat shock protein 60	1464	24
4	Heat shock 70 kDa protein 8	1367	31
5	Pyruvate kinase muscle isozyme	1243	26
6	Elongation factor 2	1225	24
7	Fanconi anemia group J protein homolog	1199	28
8	Elongation factor 1	1134	21
9	Tubulin beta-3 chain	1119	20
10	Actin, alpha cardiac muscle 1	963	17
11	Stress-70 protein	858	17
12	Alpha-enolase	835	19
13	Tubulin beta-1 chain	778	15
14	Tubulin alpha-1 chain fragment	751	12
15	Putative uncharacterized protein	692	17
16	Tubulin alpha-5 chain	614	14
17	Acetyl-CoA carboxylase beta fragment	558	15
18	78 kDa glucose-regulated protein precursor	437	12
19	pep:known chromosome:WASHUC2	436	5
20	Heat shock cognate protein HSP 90-beta	406	8

Table 3.3.3.1. Mass spectrometry profile of FANCJ Strep immunoprecipitation eluate. The FANCJ Elution 1 liquid eluate from the Strep immunoprecipitation was sent for analysis by MS. The bait protein FANCJ was successfully identified in the elution sample.

	WT		
S.No.	Name of Protein	Total number of peptides	Unique peptides
1	Biotinyl peptide fragment	4473	113
2	Actin, cytoplasmic type 5	2219	28
3	Heat shock protein 60	1670	24
4	Elongation factor 1-alpha 1	1373	20
5	Elongation factor 2	1325	29
6	Heat shock 70 kDa protein 8	1262	27
7	Tubulin beta-3 chain	1245	24
8	Pyruvate kinase muscle isozyme	1153	24
9	Tubulin beta-3 chain	1139	20
10	Actin, alpha cardiac muscle 1	939	19
11	Tubulin beta-1 chain	900	15
12	Alpha-enolase	885	22
13	Glyceraldehyde-3-phosphate dehydrogenase	881	11
14	Tubulin beta-1 chain	835	19
15	Tubulin alpha-5 chain	597	16
16	pep:known chromosome:WASHUC2	593	8
17	Tubulin alpha-1 chain fragment	532	14
18	75 kDa glucose-regulated protein	462	13
19	Acetyl-CoA carboxylase beta fragment	390	11
20	78 kDa glucose-regulated protein precursor	331	11

Table 3.3.3.2. Mass spectrometry profile of WT Strep immunoprecipitation eluate. The WT Elution 1 liquid eluate from the Strep immunoprecipitation was sent for analysis by MS as a control for the FANCJ Elution 1 liquid eluate sample.

Also, further analysis of the MS data obtained showed that the sequence coverage for the bait protein was only 39% (Figure 3.3.3.6). This was compared to the predicted peptides generated after an in-silico tryptic digest of FANCJ protein (Figure 3.3.3.7) and the peptides generated were overlapped with the obtained MS data for the Strep/FLAG tagged FANCJ elution sample. This indicated poor coverage. Immunoprecipitation should enrich the amount of bait protein in the sample and as such, there should be increased number of peptides generated after tryptic digest and as such, the sequence coverage for the bait protein should be much higher. Although, this is also dependent on how well the peptides, generated after the tryptic digest of the elution samples are able to ionize. This suggests that the bait protein is being lost during one of the immunoprecipitation steps.

Fanconi Anaemia Complementation Group J (FANCJ)

```
   1 MSSDVSQYTI GGVKIMFPCK AYPSQLAMMN AIVKGLNNRQ HCLLESPTGS
  51 GKSLALLCSA LSWQQSLYEK SLLKSSCEKE DREPAASLPC RCVCHSRSES
 101 SEATAGASHG AACSNNYETG GSVKHGDQLS DTECKENNTL ASKLSAKKRA
 151 SACGNECDDF QVERKRIRPL ETEQQVRKRH CFSKEVQLVD ALEVYNQRKN
 201 GELIVHSEKS VKNTSPQTLF SSCTECSCSS GKETRKDSGN TKKKANGDQT
 251 FIPKIFFGTR THKQIAQITR ELKRTAYSGV PMTILSSRDY TCIHPVVSSS
 301 NSNRNELCVE LLEGKHGKSC LYYHGVHKLS EHYALQSAHN TYQAWDIEDL
 351 VSLGKKLRAC PYFAARELMV GADIVFCPYN YLLDPQIRES MEINLKGQVV
 401 ILDEAHNIED SAREAVSYSV TESQLNAARE ELDFMVNNNI RQKDHEQLRA
 451 MCCSLTNWLR ESSSQLVETG YETSCKVWSG KEMLNHFHDM GITNISFPIL
 501 QKHLSAVLEK EEKISMFGKE ELVEIPIVSS ATQIVLKGLF MVLLYLFKDN
 551 SRFADDYRVA LQQTYAWTND NQPDVSDTSA FFTKTKHKRN LRHKTVVHML
 601 NFWCLNPAVA FSDLNDVRTV VLTSGTLSPM DSFSSELGVK FSIQLEANHV
 651 IRNSQVWVGT IGTGPNGRKL CATFQHTETF EFQDEVGALL LSVCQKVGQG
 701 ILCFLPSYKL LDKLKDRWIH TGLWRNLELV KTVIAEPQGG AKSDFDELLK
 751 IYYDAIKFKG EKDGALLIAV CRGKVSEGLD FCDENARAVI TIGIPFPNVK
 801 DLQVELKRKY NDQHKTTRGL LPGSQWYEIQ AYRALNQALG RCIRHRSDWG
 851 ALILVDDRFR NNPNKYITGL SKWIRQQVQH HENFGSALES LHAFAERNQK
 901 GIDFSSQCSN EVFHVPLNSK EPSSASQQEA TIHLSPDVPV KSEEQSFVPE
 951 THLTTTINSI NPGPSNQPDR CGQKVDVESC SHNGIQRRKH MDSTPRRPAN
1001 KTEKKSDRTN SDFMKEHCCF KPLTSTPLPV ATNCVSTASS KQRKNVNSAS
1051 ELIGGVNQCQ SSFTLEHKPS IPESHLETTN FSVKSTEAPV AEEHLDEQKL
1101 QIEPCSELPS VGGRPELSVL EISAEDEDES LYFTPELYDD AESEEQEMRP
1151 LDPDENQIEC GKPTVADDLF VISTSKTLSE PKEMINDDGR NTSLHGTMLS
1201 DISKNSTVNI EKMTNGEEAE QVESQEVDTK KRKISLSRSR NKGVSPFLL
```

Figure 3.3.3.6. Amino acid sequence of the identified FANCJ protein by Mass Spectrometry. The FANCJ peptides detected by Mass Spectrometry have been highlighted in red. Sequence coverage is 39%.

Start - End	Sequence
15 - 20	K.IMFPCK.A (Ions score 7)
21 - 34	K.AYPSQLAMMNAIVK.G (Ions score 79)
40 - 52	R.QHCLLESPTGSGK.S (Ions score 42)
98 - 124	R.SESSEATAGASHGAACSNNYETGGSVK.H (Ions score 96)
98 - 124	R.SESSEATAGASHGAACSNNYETGGSVK.H (Ions score 94)
125 - 143	K.HGDQLSDTECKENNTLASK.L (Ions score 89)
167 - 177	R.IRPLETEQQVR.K (Ions score 50)
167 - 177	R.IRPLETEQQVR.K (Ions score 49)
185 - 198	K.EVQLVDALEVYNQR.K (Ions score 86)
185 - 199	K.EVQLVDALEVYNQRK.N (Ions score 1)
185 - 199	K.EVQLVDALEVYNQRK.N (Ions score 59)
200 - 209	K.NGELIVHSEK.S (Ions score 49)
255 - 260	K.IFFGTR.T (Ions score 20)
264 - 270	K.QIAQITR.E (Ions score 25)
275 - 288	R.TAYSGVPMTILSSR.D (Ions score 35)
275 - 288	R.TAYSGVPMTILSSR.D (Ions score 32)
319 - 328	K.SCLYYHGVHK.L (Ions score 11)
359 - 366	R.ACPYFAAR.E (Ions score 1)
397 - 413	K.GQVVILDEAHNIEDSAR.E (Ions score 109)
414 - 429	R.EAVSYSVTESQLNAAR.E (Ions score 80)
450 - 460	R.AMCCSLTNWLR.E (Ions score 52)
503 - 513	K.HLSAVLEKEEK.I (Ions score 51)
559 - 584	R.VALQQTYAWTNDNQPDVSDTSAFFTK.T (Ions score 75)
595 - 618	K.TVVHMLNFWCLNPAVAFSDLNDVR.T (Ions score 34)
595 - 618	K.TVVHMLNFWCLNPAVAFSDLNDVR.T (Ions score 0)
641 - 652	K.FSIQLEANHVIR.N (Ions score 52)
788 - 800	R.AVITIGIPFPNVK.D (Ions score 62)
801 - 808	K.DLQVELKR.K (Ions score 33)
819 - 833	R.GLLPGSQWYEIQAYR.A (Ions score 95)
834 - 841	R.ALNQALGR.C (Ions score 25)
861 - 872	R.NNPNKYITGLSK.W (Ions score 66)
876 - 897	R.QQVQHHENFGSALESLHAFAER.N (Ions score 39)
975 - 987	K.VDVESCSHNGIQR.R (Ions score 70)
1016 - 1041	K.EHCCFKPLTSTPLPVATNCVSTASSK.Q (Ions score 58)
1045 - 1084	K.NVNSASELIGGVNQCQSSFTLEHKPSIPESHLETTNFSVK.S (Ions score
1085 - 1099	K.STEAPVAEEHLDEQK.L (Ions score 83)
1085 - 1099	K.STEAPVAEEHLDEQK.L (Ions score 31)
1183 - 1204	K.EMINDDGRNTSLHGTMLSDISK.N (Ions score 6)
1191 - 1204	R.NTSLHGTMLSDISK.N (Ions score 84)
1205 - 1212	K.NSTVNIEK.M (Ions score 30)
1213 - 1230	K.MTNGEEAEQVESQEVDTK.K (Ions score 108)

Figure 3.3.3.7. Peptides generated after trypsin digestion of FANCJ.
Tryptic digestion of FANCJ protein generated the following peptides (highlighted in red). The sample was then subject to Mass spectrometry.

Given the results above, it can be concluded that the Strep only immunoprecipitation is a good start and the immunoprecipitation protocol needs to be optimized further in order to perform a successful immunoprecipitation where MS identifies the bait protein and its known interacting partners in the elution sample. This will validate the immunoprecipitation and increase confidence in the FANCJ interacting proteins subsequently identified by bioinformatics. It is also important to have increased sequence coverage and an increased number of peptides detected by MS for each of the identified proteins as this will further increase confidence in the generated MS data.

Chapter 4: DISCUSSION

The FANCJ helicase activity is implicated in DNA replication (Shen et al 2009), repair and maintenance of genomic stability in eukaryotes (Wu and Brosh 2009). Biallelic mutations in FANCJ are associated with the chromosome instability disorder Fanconi anaemia and monoallelic mutation with the inherited predisposition to early onset breast cancer. Given the above, FANCJ is emerging as an important protein for the prevention of human diseases, such as cancer and chromosomal instability. However, its role in DNA repair and precise mechanism of action still remains unclear. Thus, in order to gain an insight into its possible function(s) in DNA repair, we decided to study FANCJ and its associated complexes.

Studying the protein-protein interactions can lead to better understanding of the biological interaction network of FANCJ and eventually the FA pathway. This will shed light on FANCJ functions in maintenance of genome stability. It might also reveal the presence of new genes involved in FA. It is equally important to know and understand how different proteins interact with FANCJ, whether proteins from various pathways can cross talk and the implications of this possible cross talk. It is equally important to understand how the interactions, localization(s) and functions are affected in the presence of mutated copy of FANCJ. It is also interesting to explore the strategies a cell employs in order to cope with normal endogenous and exogenous damage in presence of normal and mutated FANCJ protein. It is expected that the identification and characterization of these associations will provide further understanding of the role of FANCJ in DNA replication, repair and genome stability.

The most extensive studies on endogenous protein interaction networks have been performed in yeast. Here, in-locus epitope tagging of the open reading frame of the protein of interest is feasible via homologous recombination (Gari et al 2008, Seki et al 2007). The tagged proteins are under the regulation of endogenous promoters, which lowers the rate of false positives by approximately 60% (Gari et al 2008, Seki et al 2007). Tandem immunoprecipitation protocol, using double affinity tags reduces the background caused by nonspecific binding of proteins, as it combines two affinity purifications based on two different affinity matrices. The complexes isolated are of higher purity and their likely associated proteins identified (Gloeckner et al 2009). This makes the analysis of the data generated by MS much easier. Here, I used these approaches to generate the Strep/FLAG tagged FANCJ cell line in the chicken DT40 system. The use of the DT40 system has a number of advantages. DT40 is an excellent system to study molecular mechanisms of the FA pathway, because the complete set of FA genes are only found in vertebrates (D'Andrea and Grompe 2003). DT40 cells are phenotypically stable, compared to human tumor cell lines (Sale 2004) and it is easy to modify genes in DT40 cells (Buerstedde and Takeda 1991). Such cell lines provide an excellent tool to delineate gene function by reverse genetics. It is also possible to introduce an epitope tag by ‘knock-in’ into a specific gene. Additionally, DT40 cells grow very quickly, and are easy to handle. Also, a suitable antibody that demonstrates specificity and selectivity for a protein of interest enables the analysis of its protein interactions by subsequent MS analysis of the immunoprecipitated samples. There are no specific antibodies against *Gallus gallus* FANCJ. Thus, in order to study FANCJ and its interacting partners in the DT40 system, the in-situ double epitope tagged FANCJ cell line was generated. This approach to identify the interacting partners and to study the functions of FANCJ is far more sensitive and

superior to overexpression of a tagged version of FANCJ in human cells as, the MS data obtained from the immunoprecipitation of an overexpressed protein is known to have an increased non-specific background and an increased rate of false positives, which makes the analysis of the subsequent MS difficult. The in-situ double epitope tagged FANCJ DT40 cell line overcomes these problems and will allow for easier analysis of the MS data generated after an immunoprecipitation.

The tandem affinity purification tagging delivers a powerful and robust technique to purify and isolate proteins of interest and the complexes they may be a part of. The tandem affinity purification followed by specific elution steps (biotin elution and FLAG peptide elution) provides specificity and stringency at a high-throughput purification procedure. Importantly, this technique is an excellent method for identifying binding partners for a protein target of interest, as the mild tandem washing steps involved significantly decrease the non-specifically interacting proteins while maintaining all the specific interactions. With the introduction of high throughput sequencing, the focus for the efficient production of recombinant proteins has shifted towards facilitating the expression and following purification of the recombinant proteins. In order to overcome known problems of protein production, such as inefficient translation, aggregation, limited solubility, or degradation and to allow efficient purification, affinity tag systems have become an indispensable tool (Waugh 2005). Affinity tags allow single step purification or tandem purification procedures resulting in highly pure eluted bait protein. In addition, tags can reduce aggregation, increase solubility and promote proper folding, thereby increasing the yield of recombinant proteins. Besides the ubiquitous hexa-his tag, alternative tag systems have been developed over the years and all of them have different strengths and weaknesses e.g. MBP, GST, CBP, STREP, myc, FLAG (Waugh 2005). For our immunoprecipitations, we decided to use the Strep tag II and FLAG tag.

The original Strep tag (AWRHPQFGG) facilitates binding to the streptavidin matrix, but only when fused to the C-terminus of the desired protein (Schmidt and Skerra 1994). This limitation is due to a salt bridge between the carboxy-group of the free C-terminus of the Strep tag and Arg84 of streptavidin (Schmidt et al 1996). Improvement in the Strep tag system, i.e., the discovery of Strep tag II (WSHPQFEK), which is not affected by this limitation, gives much more flexibility to applications with Strep tags. An optimized streptavidin (StrepTactin) with high affinity to Strep tag II was also engineered (Voss and Skerra 1997). This novel combination of Strep tag II and the StrepTactin matrix demonstrated that the Strep tag II technique yielded 6.5 times more purified protein after a single chromatographic step compared to ion chromatography (Maier et al 1998). The Strep tag II affinity column is much easier to handle, only a few column volumes of washing buffer is sufficient to purify the sample and elution can be performed in a single step because of the high specificity of binding between StrepTactin and the elution peptide Desthiobiotin. The affinity column can be run in a short time by gravity flow without technical support and can be reused (Maier et al 1998).

The FLAG tag is one of the most commonly used systems. Hopp and co-workers first described the FLAG tag (DYKDDDDK) in 1988 (Hopp et al 1988) and it was intended to fulfill certain criteria, e.g., the tag should be as short as possible but still long enough to form an epitope for antibody recognition, it should be highly soluble, it should have minimal impact on protein folding, the sequence DDDDK was selected

to allow enterokinase cleavage of the tag. Lysine (K) in the third position was introduced to increase hydrophilicity and tyrosine (Y) was selected as aromatic residues often improve antibody binding (Hopp et al 1988). The first antibody used to purify FLAG-tagged proteins (M1) was only able to detect the FLAG tag at the N-terminus which not be preceded by other amino acids and it (M1) also showed to be Ca^{2+} dependent, allowing mild elution of bound proteins via EDTA (Hopp et al 1996) (Prickett et al 1989). This limitation led to the development of further anti FLAG monoclonal antibodies, namely M2 and M5. These antibodies were more flexible and allowed detection of the FLAG tag irrespective of the positioning of the tag, i.e., C or N terminus. These qualities have made the FLAG tag highly desirable for tagging proteins. FLAG tag has been used for numerous applications such as; protein detection and purification strategies, which include immunoprecipitation, protein-purification, and tracking of tagged proteins in cells and tissues.

From the results presented here, it can be concluded that I have developed and validated a new experimental system to investigate the role of FANCJ in DNA replication and repair. The Strep/FLAG tagged FANCJ cell lines generated will be used to identify the interacting partners of FANCJ and to define an interactome. This will be followed by the functional characterization of FANCJ and its associated proteins and yield insights into their role in DNA replication and repair. The generated Strep/FLAG tagged FANCJ cell lines were subject to proteomic analysis to check for expression of the tagged protein, to assess the availability of the Strep/FLAG tags, and the response of Strep/FLAG tagged FANCJ to increasing drug doses of cisplatin with wild-type as a positive control and FANCJ-/- (FANCJ knock-out cell line) as negative control to establish if the tags had interfered with protein function. It was established that the generated Strep/FLAG tagged FANCJ cell lines showed a phenotype similar to the wild type, which indicated that tagging had not interfered with the protein function. After characterizing the generated cell lines, Strep/FLAG tandem affinity immunoprecipitation was performed to identify Strep/FLAG tagged FANCJ and its associated proteins by MS. Although immunoblots clearly showed the presence of Strep/FLAG tagged FANCJ in the Strep/FLAG immunoprecipitation eluate, we were unable to detect it by MS analysis. MS can detect proteins in the femtogram range, therefore, it was concluded that the yield of protein(s) from the Strep/FLAG-TAP was somehow insufficient for MS. As such, a change in strategy to immunoprecipitate and identify Strep/FLAG tagged FANCJ by subsequent MS was adopted. Also, StrepTactin has a binding capacity of 5μg/μL whereas the anti-FLAG M2 agarose resin has a binding capacity of 600ng /μL. Since the binding capacity of StrepTactin is much higher and the Strep tag is a widely used tagging system because of its high affinity, speed of isolation and the abundance of material produced (Schmidt and Skerra 2007), a Strep immunoprecipitation was carried out. The MS analysis on the Strep elution sample successfully identified the bait protein but no known FANCJ interacting proteins were detected. This implies need to further optimize the Strep immunoprecipitation, in order to identify FANCJ and its associated proteins by MS.

Chapter 5: FUTURE WORK

Optimizing the Strep immunoprecipitation

Since only the bait protein and none of the know interactors were detected in Strep immunoprecipitation, which undermines the validity of the immunoprecipitation to define the FANCJ interactome, current focus lies on optimizing the protocol in order to increase the yield of the bait protein and to detect published interactors along with hopefully as of yet unknown interactors. A parallel approach to subfractionate cells and specifically purify chromatin-associated proteins in order to isolate the protein complexes they may be a part of can also be adopted. This approach enables the separation and purification of chromatin associated proteins and their interacting partners under physiological salt conditions (Aygun et al 2008) thus enriching the bait protein and its associated proteins, which will increase the possibility of detection by MS. Once the Strep immunoprecipitation has been optimized, three independent Strep immunoprecipitations will be performed followed by MS. The MS profile from these three immunoprecipitations will be overlapped to generate confidence in the proteins reproducibly identified and thus help in defining an interactome for FANCJ.

These experiments will be followed by immunoprecipitations from the tagged FANCJ cell lines grown in the presence of different drugs, e.g. CP, HU (Hydroxyurea), etc. MS on the elution samples might reveal the presence of conditional complexes.

Validation of interaction(s)

Once a comprehensive set of FANCJ immunoprecipitations has been conducted, the data identified by MS, then shall be subject to interrogation by bioinformatics. Oxford University has a Trans Proteomics Pipeline (TPP), which went live in May 2009. Software is based on Seattle Proteome Center's TPP and was implemented and further developed by David Trudgian. The TPP supports multiple search engines for MS data searching, data analysis, statistical analysis of proteomics data and tools for quantitative proteomics, in order to identify more proteins from a complex mixture than Mascot alone and to narrow the number of proteins subject to further analysis. Bioinformatics support is provided by the Computational Biology Research Group (CBRG). CBRG hosts a MASCOT server for MS data searching. First of all, bioinformatics will be used to identify the proteins reproducibly present only in the FANCJ immunoprecipitations and not in the wild-type control. Secondly, information regarding nuclear compartmentalization, presence of conserved domain(s) or domain(s) implicated in DNA metabolism and repair, previously known activity in DNA replication or repair; will be sought for the FANCJ interacting protein(s). This is important for validating the interaction and will help in understanding the possible role of the interaction(s).

Bioinformatics will help narrow down the list of FANCJ interacting protein(s), which will be subject to further validation of interaction and functional studies.

Co-Immunoprecipitation

The FANCJ interacting protein(s) identified after the bioinformatics analysis of the MS data generated by the three Strep immunoprecipitations will be validated by Co-immunoprecipitations. The interacting protein(s) will be immunoprecipitated and then immunoblotted with antibodies against the Strep or FLAG tags of the Strep/FLAG tagged FANCJ to confirm that these proteins are interacting partners. If antibodies are not available for these identified protein(s), then they will be cloned, tagged and endogenously expressed and then subject to immunoprecipitations followed by immunoblotting to confirm the given interaction.

Functional analysis

Once these interaction(s) have been validated, reverse genetic studies on DT40 cells by disrupting genomic loci of the identified interacting partner(s) or by using siRNAs in human tumor cell lines to knockdown protein function, will generate information about whether knock-out or knockdown of the protein interactor(s) generates a phenotype similar to FANCJ knock-out cell line. Further, changes in γH2AX foci formation, FANCD2 and FANCI monoubiquitination status, changes in recombination status will be investigated. Analyzing response to different DNA damaging compounds will follow this. It will be interesting to see whether the knockout or knock down of the FANCJ interacting proteins(s) increases or alleviates the sensitivity of FANCJ deficient cells to the DNA damaging compounds. This will shed light on the possible function(s) of the new interacting partner(s) and help in defining the cellular pathways FANCJ may be a part of and its possible mode of action. Different FANCJ domains will also be cloned, tagged and expressed endogenously, followed by immunoprecipitations. This will assist in the identification of the binding site(s) of the identified protein(s) on FANCJ and confirm the interaction between FANCJ and the identified protein(s) as well. It will be interesting to see whether mutations in these binding sites are present in FA-J patients and what possible functions/pathways are being disrupted because of these mutations. Further, the different FANCJ mutants defective for specific interactions can also be cloned, tagged and endogenously expressed in order to study the role of their interaction(s) and the effect(s) these mutations have on DNA repair, in response to different DNA damaging compounds. Single, double or even triple mutants can be generated with the help of different selection markers. Mapping the domains of FANCJ and their interactions will be important for separation of function studies. These shall also provide mechanistic insight into the FANCJ associated DNA repair.

Study of Post Translational Modifications

Alternate strategies for studying the role of FANCJ in DNA repair can also be adopted. A study on the different PTMs of Strep/FLAG tagged FANCJ, using the StrepII tag under denaturing conditions will also be done. It shall be interesting to study the type and role of PTMs in modifying the functions of FANCJ during replication and repair. Endogenous FANCJ is phosphorylated in response to cellular exposure to TMS (telomestatin) (Wu et al 2008). FANCJ phosphorylation was also observed when cells were subjected to UV (Garner and Smogorzewska 2011b), suggesting a mechanism for the modulation of FANCJ function during the DNA

damage response. The phosphorylation state of FANCJ is also important for functions other than the response to exogenous DNA damage, since FANCJ is silenced during the G1 phase of the cell cycle and is activated through a dephosphorylation event as cells enter S phase (Matsushita et al 2005).

Study of the role of different FANCJ domains in DNA repair

The different FANCJ domains can be cloned and tagged. These tagged domains can be then expressed in cell lines to identify their specific interacting partners and to understand the role these domains in DNA repair. Introduction of mutated FANCJ domain(s) can also help increase our understanding of the role of FANCJ in DNA repair.

These experiments will help elucidate the role FANCJ and its associated proteins in DNA repair and maintenance of genome stability and eventually increase our understanding of the relationship between DNA repair and human disease.

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Appendix

MATERIALS

Equipment

Centrifuges

Cell culture centrifuge (Eppendorf, 5702)
Cell culture centrifuge (Sorvall legend RT)
Cooled microfuge (Eppendorf, 5415 R)
Medium speed centrifuge (Beckman coulter; JA-20, JA-14)
Tabletop centrifuge (Eppendorf, 5424)

Cold storage

-80 ^{o}C ultra-freezer (Beynon West, CB5355-3)
Liquid nitrogen cell storage tank
Dry ice

Cell culture

Cell culture incubator (Kendro laboratory products, BB6220)
Cell culture sterile hood (Advanced biosafety cabinet class 2, Heraeus)
Cell counting chamber (Neubauer system)
Cell freezing box (Styrofoam)
Inverted microscope (Olympus CK2)
Vacuum pump for washing cells (CAPEX 2DC)
Waterbath (Techne, TE8J)

Mixed other equipment

Bacterial shaker (Innova 44, Newbrunswick scientific)
Electroporator for stable targeted integration/transfection (BioRad Gene Pulser II)
Electroporator for transient transfection (Amaxa Biosystems – Nucleofector II)
Hotblock (Techne dry block, DB-2D)
Ice machine (Ziegra, SG200)
Incubator for bacterial plates (Thermoscientific D-63505, Type B15)
Gel electrophoresis chamber, large (BioRad)

Gel electrophoresis chamber, small (BioRad)
Rotator (Stuart, SB3)
Paper cutter (Precision cutter, 640N)
pH meter (Metter Toledo, FE20/FG2)
Photometer (DNA – NanoDrop, ND1000)
Photometer (Protein – Biotek instruments, μQuant)
Power supply (BioRad)
Scale (AND, EK200i)
Scale for minute amounts (Denver instrument, S1-234)
See saw rocker (Stuart, SSL4)
Sonicator (Soniprep, 150)
Transilluminator (Alpha Innotech Alpha imager HP Multi Image II)
Vortex (Starlab wizxard)
Water purification system (Millipore, CDUFBI001)
X-ray film developer machine (Xograph imaging systems)

Consumables

1.5 mL microcentrifuge tubes, sterile (Axygen, MCT-150-C)
15 mL tubes with lid, sterile (Corning, 430790)
50 mL tubes with lid, sterile (Corning, 430829)
Bacterial culture tubes (BD Falcon Ref. 352059)
Cell culture flasks with filter lid, sterile, 25 cm^2 (Corning Flask, 430639)
Cell culture flasks with filter lid, sterile, 75 cm^2 (Corning Flask, 430641)
Cell culture flasks with filter lid, sterile, 175 cm^2 (Corning Flask, 431080)
Cryo vials, sterile, 2 mL (Corning, 430489)
GenElute HP Endotoxin Free Maxiprep Kit (Sigma, Cat. no. NA0410-1KT)
GeneJet Plasmid miniprep kit (Fermentas, Cat. no. K0503)
Hybond-C Extra Nitrocellulose membrane (Amersham Biosciences)
Medical X-ray films (FUJIFILM 47410 08389)
Plastic pipettes, sterile, 5 mL (BD Falcom Ref. 357543)
Plastic pipettes, sterile, 10 mL (Corning, 4488)
Plastic pipettes, sterile, 25 mL (Corning, 4489)
Plastic pipettes, sterile, 50 mL (Corning ,4490)

Cell culture media

Growth media

RPMI1640 (+ L Glutamine, Gibco Ref. 21875-034)
Fetal Calf Serum (FCS - PAA, A15-151)
Chicken Serum (CS - Sigma, C5405-500mL)
Penicillin + Streptomycin (PAA, P11-010, 100 x stock)
β-Mercaptoethanol (Sigma, M3148-100mL)

Reagents and Chemicals

Affinity Matrices

Anti-FLAG M2 agarose resin (Sigma, A220-1mL)

FLAG peptide (Sigma, F3290-4MG)
Strep-Tactin Superflow 50% suspension (IBA Cat. no. 2-1206-010)
Desthiobiotin (IBA Cat. no. 2-1000-002)

Antibiotics

Ampicillin (Sigma, A0166-25G)
Blasticidin (InvivoGen Cat. no. ant-bl-1)
Neomycin (Sigma, A1720-5G)

Detergents

Tween 20 (Sigma, P9416-100ML)
Nonidet P-40 (Igepal-CA-630; Fluka 56741))
SDS (Sigma, L4390-500G)

Dyes

Bromophenol blue (Sigma, B0126-25G)
RedSafe (Intron, 21141/0008-090302142)
Xylene cyanol (Sigma, X4126-10G)

Enhanced Chemiluminescence (ECL) Detection

Immobilon Western (Millipore Cat. no. WBKLS0500)

Enzymes

BamH1 (Fermentas, ER0051)
Benzonase (Novagen, 70746 10KUN)
EcoR1 (Fermentas, ER0271)
Not1 (Fermentas, ER0592)
T4 DNA Ligase (New EnglandBiolabs, M0202S)

Molecular weight standards

Blue wide range protein ladder (Cleaverscientific, CSL-BBL)
O'Generuler 1kb DNA Ladder (Fermentas, SM1163)
Protein standard (Novex, P/N 57318)
Zipruler express DNA ladder set (Fermentas, SM1373)

Protein phosphatase Inhibitor

PhosSTOP (Roche, 04 906 837 001)

Protease Inhibitor

Complete EDTA free (Roche, 05 056 489 001)

Miscellaneous

Acrylamide/Bis-acrylamide solution (BioRad Cat. no. 161-0156)
Agarose (Invitrogen Cat. no. 16500-500)
AlamarBlue (Invitrogen, DAL1025)
Ammonium persulfate (Fisher, A/P470/40)
Bicine (Melford, B7406)
Bovine serum Albumin for Bradford assay standard (Roche, 10 735 086)
Bradford reagent (BioRad Cat.no. 500-0006)
Copper sulfate (Prothermal Cat. no. 2601)
DMSO (Sigma, D8418-250mL)
Ethanol (Fisher, E/0650DF/P17)
Glycine (Melford, G0709)
Glycerol (Invitrogen, 1554-029)
Hydrochloric acid (Fisher, H/1200/PB17)
Manganese(II) chloride (Sigma, M8054-100G)
β-Mercaptoethanol (Sigma, M3148-100mL)
Methanol (Fisher, M/3950/17)
Milk powder (Marvel)
NaCl (Sigma, S7653-5Kg)
Isopropanol (Fisher, P/7500/PC17)
TrisBase (Melford, B2005)
TEMED (Sigma, T9281-50mL)
Urea (Sigma, 451-1kg)

Antibodies and detection

Antibodies for protein detection in Western Blot

Antigen	Host species	Source	References and comments
FLAG	Mouse	F 1804, Sigma	1:1000 in 5% milk
Strep	Rabbit	PK-AB718-4335, Promokine	1.5:5000 in 5% milk
Strep (HRP-coupled)	Rabbit	2-1509-001, IBA	1:5000 in 5% milk
TOPBP1	Rabbit	A300-111A, Bethyl	1:5000 in 5% milk

Horse radish peroxide (HRP)-coupled secondary antibodies for ECL

Antibody	Source and comments

HRP-coupled anti-rabbit IgG	PO448, Dako, 1/5000 in 5% milk
HRP-coupled anti-mouse IgG	PO447, Dako, 1/2000 in 5% milk

Plasmids: Targeting vectors and cloning vectors

Targeting vector backbone

pBluescriptKS+/- (Fermentas)

Cloning vector

pCR 2.1-Topo (Invitrogen, TopoTA cloning kit Dual promoter pCRII Topo Vector Part 45-0640)

Cell lines

Cell line	**Stable targeted knock-in insert**	**Growth medium**
DT40	Wild type	RPMI1640 + FCS 7% + CS 2% + 100μg/mL of penicillin + 100μg/mL streptomycin + 0.01% β-Mercaptoethanol.
DT40	C-terminally Strep and FLAG tagged FANCI	RPMI1640 + FCS 7% + CS 2% + 100μg/mL of penicillin + 100μg/mL streptomycin + 0.01% β-Mercaptoethanol.
DT40	C-terminally Strep and FLAG tagged FANCJ	RPMI1640 + FCS 7% + CS 2% + 100μg/mL of penicillin + 100μg/mL streptomycin + 0.01% β-Mercaptoethanol.

Competent Bacteria

DH5α (plasmid-free strain of chemically competent *E.coli* present in the laboratory).

Bacterial stock

DH5α (containing C-terminally Strep and FLAG tagged FANCJ targeting vector with Neomycin resistance cassette).

SOLUTIONS AND BUFFERS

Bacterial culture

LB medium (provided by Cancer Reseach UK central media services)

10g Bacto-typtone
5g Bacto-yeast extract
10g NaCl

Dissolve in 950 mL H_2O and adjust pH 7.0 with 5M NaOH. Fill upto one litre and autoclave.

LB agar plates with selection (provided by Cancer Research UK central media services)

Microwave LB-agar (LB medium solidified with 15g/L agar) in a bottle with slightly opened lid under repeated mixing until fully resolved. Cool to 45 ^{o}C and add antibiotics as required. Mix and pour into plates. Let the plates cool down and store in sealed plastic bags upside down at 4 ^{o}C for upto 4 weeks.

2.3.2 Eukaryotic cell culture

Growth medium

500 mL RPMI1640 (provided by CRUK central media services)
38 mL Fetal Calf Serum (provided by CRUK central media services)
12 mL Chicken Serum
5 mL Penicillin + Streptomycin
500 μL β-Mercaptoethanol

Cell freeze medium

31.2 mL FCS
10.4 mL CS
6.4 DMSO

Medium was prepared fresh every time before use. 1mL of freezing medium was used for freezing 1 x 10^6 upto 5 x 10^6 cells.

10 x PBSA (phosphate buffered saline; provided by Cancer Research UK central media services)

800g NaCl
25g KCl
143g Na_2HPO_4
25g KH_2PO_4
Dissolve in 10 litre H_2O.

2.3.3 Cell lysis and protein analysis

1 x Bradford protein assay reagent

To 4 ml of 4 x Bradford reagent, add 20 mL H_2O and mix immediately. Store at 4 ^oC until use.

5x SDS-PAGE protein gel sample loading buffer (Lammeli buffer)

Blocking buffer for Western blots

1 x TBS
1% Tween 20
5% Low fat dry milk

Dissolve in H_2O and store at room temperature.

BSA solution for Bradford assay

Dissolve 1mg/mL BSA in H_2O (total 100 mL solution), aliquot into 500μL aliquots and store in -20 ^oC.

FLAG elution buffer

30mM Tris
150mM NaCl
400μg/mL FLAG peptide

5X Lamelli Buffer

625mM Tris-HCl pH 6.8
5% SDS
25% glycerol
100mM β-mercaptoethanol
0.025% Bromophenol blue)

Aliquot into 500μL aliquots and store in -20 ^oC.

Lysis buffer

30mM Tris
150mM NaCl
0.25% NP-40
1mM Ca^{2+}
Supplemented with protease and phosphatase inhibitors

Buffer was prepared fresh every time before use.

Lower buffer

1.5M Tris.Cl pH 8.8
0.4% SDS

Running buffer – Tris Bicine gels)

100mM TrisBicine
0.2% SDS

SDS-PAGE buffer

250mM Tris
1.9M Glycine
1% (w/v) SDS

Dissolve in H_2O and store at room temperature.

SDS-PAGE gels

Seperating gel (8%)

8.5 mL Acrylamide
15.2 mL H_2O
8 mL Lower buffer
120 µL 10% APS
60 µL TEMED

Stacking gel (4.5%)

1.5 mL Acrylamide
6 mL H_2O
2.5 mL Upper buffer
50 µL 10% APS
40 µL TEMED

Strep elution buffer

30mM Tris
150mM NaCl
2.5mM Desthiobiotin

Buffer was prepared fresh every time before use.

TBS

30mM Tris pH 7.5
150mM NaCl

TBST

50mM Tris base
150mM NaCl pH 7.5
0.1% Tween-20

Transfer buffer (Western Blot – SDS- PAGE gels)

86.4 g Glycine
18 g Tris
1.2 L Methanol

Dissolve in 4.8 L H_2O and use.

Transfer buffer (Western Blot – Tris bicine gels)

25mM Tris base
192mM Glycine
20% (v/v) Methanol

Tris/Bicine (1M)

121 g Tris
163 g Bicine

Dissolve in 1 L H_2O and use. Store at room temperature.

Tris HCl (provided by Cancer Research UK central media services)

121.1 g Trizma base

Dissolve in 1 L H_2O and adjust pH to 6.8, 7.5 or 8.8 and store at room temperature.

Tris bicine gels (7%)

30 mL H_2O
4.5 mL Tris/Bicine (1M)
10.5 mL Acrylamide
250 μL 20% SDS
250 μL TEMED
250 μL 10% APS

Upper buffer

0.5M Tris.Cl pH 6.8
0.4% SDS

Urea buffer

9M Urea
50mM Tris-HCl pH7.5
150mM β-mercaptoethanol

Dissolve in MilliQ. Once dissolved, filter-sterilize and store at room temperature.

Wash buffer

30mM Tris
150mM NaCl,
0.1% NP-40
Supplemented with protease and phosphatase inhibitors

Buffer was prepared fresh every time before use.