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IDENTIFICATION OF NOVEL FANCD2 INTERACTING PROTEINS VIA IMMUNOPRECIPITATION AND MASS SPECTROMETRY

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IDENTIFICATION OF NOVEL FANCD2 INTERACTING PROTEINS VIA IMMUNOPRECIPITATION AND MASS SPECTROMETRY

BY

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ABSTRACT

Fanconi Anemia (FA) is a rare genetic disease caused by biallelic mutations in one of sixteen genes involved in the FA-BRCA DNA damage repair pathway. The proteins encoded by these genes function cooperatively in a common pathway which resolves lesions caused by interstrand crosslinks (ICLs). A critical step in this pathway is the monoubiquitination and chromatin targeting of FANCD2 and FANCI. The mechanism by which these proteins are targeted to chromatin is not understood. FANCD2 is known to interact with several downstream proteins while associated with chromatin. Finding new FANCD2 interacting proteins is critical to understanding how FANCD2 functions and how it is regulated within the cell. I have identified several candidate interacting proteins by immunoprecipitations (IPs) coupled with mass spectrometry. Candidates include transcription factors, chromatin remodeling complex components and proteins involved in chromosome maintenance and stability. These interactions are being validated and functionally characterized using a variety of techniques.

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I want to thank everyone who helped me on the way to completing my thesis project as well as the many other projects I was able to work on during my time at URI. My mentor Dr. Niall Howlett has been instrumental, and I thank him for allowing me to work in his lab and learn many techniques while pursuing a deeply interesting research topic. His insight has pushed me to hone my strengths and helped me make difficult decisions. I would like to thank Dr. Steven Irvine for allowing me to work in his lab as an undergraduate and serving on my committee. I would also like to thank Dr. Ying Zhang for serving on my committee. Thanks to Dr. James Clifton for his help at Brown's EPSCoR facility. Thanks to Dr. Maurizio Mauro and Dr. Ananda Mukherjee for their input in the Howlett lab. Special thanks to Dr. Meghan Rego who patiently taught me most of the basic techniques used in the Howlett lab. Thanks to my lab members Karissa Paquin, David Vierra, Elizabeth Vouno and Rebecca Boisvert who worked with me on several other projects during my time in the Howlett lab. Thanks to my family, particularly Paul and Kathy Azzinaro, my parents, for their continued support in a long and nonlinear path of professional development. Thanks to my friends Chris Fiore and Kevin Sherman and Ted Spinard for help in unexpected places. Special thanks to my significant other Rhiannon Ketchem for maintaining our mutual survival through very stressful times.

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STATEMENT OF THE PROBLEM

FANCD2 is a critical protein in the FA pathway, which is monoubiquitinated by the core complex and localized to chromatin. Abrogation of either of monoubiquitination or chromatin localization results in the defective repair of damage caused by interstrand crosslinks. Both the regulation and function of FANCD2 are poorly understood. Identification of novel FANCD2 interacting proteins is critical to fully understanding the role that FANCD2 plays in the cell. The goal of the project is to identify novel FANCD2 interaction protein candidates using immunoprecipitation in tandem with mass spectrometry and then to validate and functionally characterize these interactions. The hope is that this data may shed light on the biochemical process in the cell and open new opportunities for patient treatment.

CHAPTER 1

INTRODUCTION

Fanconi Anemia

Fanconi anemia is a rare genetic disease characterized by congenital defects, genomic instability, and a predisposition to bone marrow failure and cancer (Walden 2014). Biallelic mutations in one of sixteen *bona fide* FA genes cause this disease. These gene products act cooperatively in the FA-BRCA pathway to recognize and repair ICLs within the DNA (Walden 2014). ICLs are highly genotoxic complex lesions which prevent DNA strand separation required for both replication and transcription (Deans 2011). The inability to correctly repair these lesions may lead to replication fork stalling or arrest, deleterious repair, or cell death particularly in white blood cells (Deans 2011). Most FA patients present anemia and early onset of bone marrow failure. Hypersensitivity to DNA crosslinking agents is a hallmark of FA patient cells (Kim 2012). This combination of increased propensity for hematological abnormalities and cancers, with fewer effective treatment options, makes the understanding of the molecular pathology critical for development of new treatment opportunities.

FA Activation

Much work has been done in recent years to help elucidate the mechanistic action of the FA proteins. The recognition and stabilization of the lesion is accomplished by FANCM, FAAP24, MHF1, and MHF2, which prevent replication

fork collapse (Meetei 2005, Ciccia 2007, Zhijiang 2010). Following lesion recognition the core complex is recruited which is composed of FANCA, FANCB, FANCC, FANCE, FANCF, FANCF, FANCG, FANCL, and FANCM. The core complex monoubiquitinates FANCD2 and FANCI on K561 and K523 respectivly, which leads to chromatin targeting and the recruitment of downstream DNA repair proteins (Garcia-Higuera 2001, Sims 2007, Smogorzewska 2007). The monoubiquitination is seen as a marker of pathway activation. The loss of any core complex member, other than FANCM, abolishes FANCD2 and FANCI ubiquitination (Bakker 2009).

FANCD2

FANCD2 is an orphan protein which has until recently been largely uncharacterized. The closest protein to FANCD2 is its paralog FANCI, which together form a heterodimer known as the ID2 complex. FANCD2 has four recognized domains, the EDGE, PIP, CUE, and NLS domains (Montes de Oca 2005, Howlett 2009, Rego 2012, Boisvert 2013). FANCD2 has also been implicated in binding directly to chromatin though the mechanism for this remains unknown. Different forms of DNA, such as circular dsDNA, dsDNA, fragments and, ssDNA, have been shown to specifically increase the association between chromatin and FANCD2 (Sareen 2012). It has also been proposed that FANCD2 harbors both nucleosome chaperone activity and the ability to promote site-specific transcriptional activation (Sato 2012, Park 2013). Despite these domains being characterized the functional and mechanistic role of this protein is still largely unknown.

FANCD2 Monoubiquitination

The monoubiquitination of FANCD2 and FANCI has been used as a marker for activation of the FA-BRCA pathway. The monoubiquitination event is key for the recruitment of FANCD2 and FANCI to chromatin. FANCD2 mutants that harbor a K561R mutation are not able to become ubiquitinated and do not rescue sensitivity to crosslinking agents (Garcia-Higuera 2001). The primary function of the FA core complex is this ubiquitination reaction. The ubiquitination is mediated through the E3 ligase activity of FANCL and UBE2T, an E2 ubiquitin conjugating enzyme specific for FANCL (Meetei 2003, Machida 2006). This ubiquitination is easily measurable by western blotting as the ubiquitin conjugation causes a detectable shift in the masses of FANCD2 and FANCI. FANCB, FANCL and FAAP100 have been shown to form a subcomplex which is able to ubiquitinate FANCD2 *in vitro* indicating that the other core complex members have some other unknown functions which are required for efficient ubiquitination *in vivo* (Rajendra 2014). This ubiquitination conjugation is a reversible reaction using the deubiquitinating enzymes USP1 and UAF1 (Nijman 2005, Cohn 2007).

FA and DNA Repair

The monoubiquitination event of FANCD2 and FANCI leads to the eventual recruitment of downstream repair proteins. The downstream proteins consist of FANCD1/BRCA2, FANCJ/BRIP1, FANCN/PALB2, FANCO/RAD51C, FANCP/SLX4, and FANCQ/XPF. It was originally thought that FANCP/SLX4 and FAN1 were recruited to the sites of damage through a specific interaction with

ubiquitinated FANCD2, however it is now known that the nucleases are recruited through a mechanism independent of FANCD2 monoubiquitination (Kimiyo 2011, Kim 2011, Shereda 2010, Chaudhury 2014, Lachaud 2014). FANCP/SLX4 acts as a recruitment platform for the nuclease complexes XPF-ERCC1, MUS81-EME1 as well as SLX1, which are able to contribute to resolving the complex structure generated by the ICLs (Zhang 2014). The incisions created by the nucleases are critical for crosslink unhooking and enabling initiation of translesion synthesis (TLS) as well as double strand breaks (DSBs) formation (Walden 2014).

TLS and Homologous Recombination

Once the crosslink has been unhooked TLS polymerases are recruited to the site to bypass the lesion. The TLS polymerases are able to synthesize across the lesion and allow for resumption of normal replication (Sharma 2013). FANCC has been shown to promote the recruitment of factors involved in switching to the error prone TLS replicative mechanism (Niedzwiedz 2004). Once the ICL lesion has been synthesized over by TLS, the DSBs that were created during nucleotide excision must be repaired in an error free manner to avoid the loss of genetic material and an increase in genomic instability. The remaining downstream proteins in the FA-BRCA pathway FANCD1/BRCA2, FANCJ/BRIP1, FANCN/PALB2, and FANCO/RAD51C promote RAD51 loading onto single stranded DNA. RAD51 is critical to the homologous recombination (HR) pathway by its ability to coordinate with a homologous DNA sequence and promote strand invasion (Mazón 2010). This process prevents the loss of genetic information by using the paired strand as a template for the

damaged strand ensuring accurate hybridization across the previously damaged region. FA patients are thought to have difficulty in promoting HR over nonhomologous end joining (NHEJ) repair which, in contrast, is an error prone mechanism. The deregulation of this error prone repair pathway has been implicated as a possible cause for the genomic instability found in FA patients (Adamo 2010, Pace 2010, Bunting 2010). It is important for patient treatment that both the mechanics and regulation of the FA-BRCA pathway are well understood.

Unbiased Screening for FANCD2 Interactors

The discovery of novel FANCD2-interacting proteins is essential for a more complete understanding of the role that FANCD2 plays in the cell. Using a variety of methods FANCD2 has been shown to interact with several proteins including, but not limited to, FANCI, FANCE, FANCJ/BRIP1, MEN1, CtIP, and the MCM helicase proteins (Sims 2007, Smogorzewska 2007, Pace 2002, Gordon 2003, Chen 2014, Jin 2003, Murina 2014, Unno 2014, Lossaint 2013). Biochemical based studies have resulted in a huge increase of the understanding in how FA works. The first six FA genes were identified through a positional cloning approach (Lo Ten Foe 1996, Fanconi anaemia/Breast cancer consortium 1996, Strathdee 1992, Timmers 2001, de Winter 1998, de Winter and Rooimans 2000, de Winter and Léveillé 2000). Biochemical approaches such as co-immunoprecipitations (co-IPs) have been instrumental in the discovery of the ten most recently discovered FA genes as well as several interacting partners (Meetei 2004, Howlett 2002, Dorsman 2007, Levran 2005, Litman 2005, Levitus 2005, Meetei 2003, Meetei 2005, Xia 2007, Reid 2007, Vaz

2010, Stoepker 2011, Bogliolo 2013). Identifying interacting networks and partners such as BRCA and BLM has provided a great deal of understanding about how the FA-BRCA pathway functions and what it is responsible for within the cell (Howlett 2002, Pichierri 2004). Immunoprecipitation coupled with mass spectrometry is a proven technique within the FA field, contributing directly to the discovery of FANCN/PALB2 (Xia 2006). Similar techniques were recently used to identify the interaction between FANCD2 and the MCM helicases (Lossaint 2013). Using large scale systems combined with the sensitivity of modern biochemical techniques we hope to discover novel FANCD2 interacting proteins using an unbiased system.

CHAPTER 2

METHODOLOGY

Cell Culture

HeLa cells (human cervical cancer cells, Scherer 1953) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco11965-092) supplemented with 12% fetal bovine serum (FBS, Gibco 26140-079) 2 mM L-glutamine (Gibco 25030-081) 500 units/mL penicillin, and 500 µg/mL streptomycin (Gibco 15070-063). PD20 (FA-D2 patient fibroblasts) cells were purchased from Coriell Cell Repositories (Catalog ID GM16633).

PD20 cells are a human transformed cell line that have a heterozygous FANCD2 deficiency as a result of biallelic mutations and lack functional FANCD2. The maternal allele contains a frameshift mutation resulting in a severe truncation, and the paternal allele has a missense mutation, causing the cells to express highly diminished amounts of FANCD2 protein which fail to correct ICL sensitivity (Timmers 2001). The corrected cells we generated using pLenti6.2-FANCD2 and pDEST40-FANCD2-V5-6xHis. The pLenti6.2 construct incorporates a V5 epitope tag on the protein which can be targeted by commercially available antibodies and a blasticidin resistance so that cells can be cultured in media supplemented with 2µg/mL blasticidin (Invitrogen R210-01) for selection. The control lines have a stably incorporated pLenti6.2-LacZ-V5 cultured with 2µg/mL blasticidin (Invitrogen R210-01) or, empty pMMP plasmid and is cultured in media supplemented with 1 µg/mL

puromycin (Sigma Aldrich P-8833). PD20s were grown in DMEM (Gibco11965-092) supplemented with 15% FBS (Gibco 26140-079) 2 mM L-glutamine (Gibco 25030-081) 500 units/mL penicillin, and 500 µg/mL streptomycin (Gibco 15070-063).

MCF10A (nonmalignant epithelial cells, Cohet 2010) cells containing doxycycline inducible SMARCA2/BRM, SMARCA4/BRG1 (members of the SWI/SNF complex) and scrambled shRNA cassettes were a generous gift from Anthony Imbalzano, and Jeffrey Nickerson with help from Karen Imbalzano, and Jason Dobson. MCF10A cells were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12, Gibco 11330-032) supplemented with 5% horse serum (Gibco 16050-122), 100 mg/mL human epidermal growth factor (EGF, Peprotech AF-100-15), 1 mg/mL hydrocortizone (Sigma Aldrich H-0888), 1 mg/mL cholera toxin (Sigma Aldrich C-8052), 10 mg/mL insulin (Sigma Aldrich I-1882), 500 units/mL penicillin, and 500 µg/mL streptomycin (Gibco 15070-063). Induction of shRNA expression was achieved by treating with 0.01µg/mL of doxycycline (Sigma Aldrich D-9891) for 72 hours and confirmed by GFP expression.

Cells were cultured at 37^{o}C with 5% CO_2 and subjected to 0.05% trypsin EDTA dissociation solution (Gibco 25300-054) for maintenance. Cells were treated with mitomycin C (MMC, Sigma Aldrich M-0503) to induce crosslink formation.

Cellular Fractionation

Cells underwent a subcellular fractionation **t**o enrich for chromatin associated proteins. Following treatment, cells were collected in 0.05% trypsin EDTA dissociation solution and DMEM, and pelleted in a centrifuge at 1200x*g* at 4^{o}C over 4

minutes. The pellet was resuspended and washed in phosphate buffered saline pH 7.4 (Gibco 10010-023) and pelleted again. Cells were first lysed in ice cold cytoskeletal (CSK) buffer (10 mM PIPES pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5% v/v Triton-X-100) for 10 minutes at 4^{o}C. The remaining pellet was washed once with CSK buffer and subjected to either nuclease reaction buffer (NRB) (20 mM Tris HCl, 100 mM KCl, 2 mM $MgCl_2$, 1mM $CaCl_2$, 0.3M Sucrose, 0.1% v/v Triton-X-100, Roche protease inhibitor cocktail tablet) or ice cold ATM lysis buffer (Sun 2009) (20 mM HEPES pH 7.4, 150 mM NaCl, .2% Tween20, 1.5 mM MgCl, 1 mM EGTA, 2 mM DTT, 50 mM NaF, 500 µM Na_3O_4V, 1 mM PMSF, Roche protease inhibitor cocktail tablet). The sample was subjected to sonication for ten seconds at 10% amplitude using a Fisher Scientific Model 500 Ultrasonic Dismembrator then supplemented with 0.1% v/v micrococcal nuclease (New England Biolabs M0247S) for twenty minutes at room temperature or 0.05% v/v benzonase (Sigma Aldrich E-1014) for thirty minutes on ice, respectively. Samples were spun at 16100x*g* for two minutes and the supernate containing the chromatin associated proteins were placed in a new ice cold tube. Chromatin fractions were quantified using the bicinchoninic acid (BCA) assay (PI-23227, Fisher) to allow for normalization.

Antibodies

Mouse monoclonal antibodies used were α-tubulin (MS-581-P1, NeoMarkers), FANCD2 (sc-20022, Santa Cruz), GFP (sc-9996, Santa Cruz), H4 (L64C1, Cell Signaling), and V5 (R960-25, Invitrogen).

Rabbit polyclonal antibodies used were CAPD2 (A300-601A, Bethyl), CAPD3 (A300-604A, Bethyl), FANCD2 (NB100-182, Novus Biologicals), H2A (07-146, upstate), H3 (ab1791, Abcam), SMC1A (A300-055A, Bethyl), SMC4 (A300-064A, Bethyl), SMC6 (A300-237A, Bethyl), and VCP (ab109240, Abcam).

Immunoprecipitation

FANCD2 immunoprecipitations were performed with protein G Sepharose (17-6002-35, GE) or protein G Dynabeads (10003D, Invitrogen). Beads were washed three times for 5 min in NETN100 (20 mM Tris-HCl, 100mM NaCl, 1 mM EDTA, 1mM Na_3O_4V, 1 mM NaF, Roche protease inhibitor cocktail tablet) + 1% bovine serum albumin (BSA) (BP1600-100, Fisher) then once more for 15 min to block the beads. The beads are washed three times in NETN100 buffer. NETN100 is added to the chromatin fraction to *q.s.* the volume up to 1 mL. A portion of the pre-blocked beads are added to the mixture containing the nuclear fraction for thirty minutes while nutating at 4^oC, to pre-clear any nonspecific interactions from the mixture. Using gentle centrifugation (or a magnet for Dynabeads) allows the beads to be separated from the mixture which is removed and placed into a new tube where the FANCD2 antibody is added and allowed to incubate while nutating at 4^oC. The beads are added to the reaction and allowed to incubate for an additional two hours while nutating at 4^oC. Gentle centrifugation (or the magnet) is used to sequester the beads at the bottom of the tube while the unbound supernatent components are aspirated. The beads are washed four times in NETN100. The beads are eluted with 2x LDS (NP-0008, NuPage) with 5% v/v β-mercaptoethanol with heating at 95^oC for 10 min.

V5 Agarose Immunoprecipitation

V5 immunoprecipitations were performed with V5 conjugated agarose beads (ab1229, Abcam). Beads were washed three times for five minutes in NETN100 (20 mM Tris-HCl, 100mM NaCl, 1 mM EDTA, 1mM Na_3O_4V, 1 mM NaF, Roche protease inhibitor cocktail tablet) + 1% bovine serum albumin (BSA) (BP1600-100, Fisher) than once more for fifteen minutes to block the beads. The beads are than washed three times in NETN100 buffer. Samples were brought up to an equal volume using the ATM lysis buffer. V5 conjugated beads were added and allowed to incubate for two hours at 4^{o}C while nutating. Beads were separated from the supernatant by gentle centrifugation. The supernatant was aspirated and the beads were washed four times with ice cold NETN100 for five minutes. After the final wash the beads were aspirated dry using a needle. The beads are eluted into 2x LDS (NP-0008, NuPage) with 5% β-mercaptoethanol by heating at 95^{o}C for fifteen minutes.

SDS-PAGE

Electrophoresis was accomplished using NuPAGE 3-8% Tris-Acetate and 4-12% Bis-Tris polyacrylamide gels (EA0378BOX and NP0335BOX, NuPAGE). Samples were lysed by either CSK buffer, NRB, ATM lysis buffer or in 2% SDS lysis buffer (SDS) (2% v/v SDS, 50 mM Tris-HCl pH 7.4, 10 mM EDTA). Lysates were supplemented with 4x LDS (NP-0008, NuPage) to a 1x concentration except for IP eluates which were run in their 2x elution buffer. Samples were run with the

Kaleidoscope prestained standard (161-0324, Bio-Rad) for reference as a molecular weight marker.

Immunoblotting

For immunoblotting analysis proteins were transferred from the SDS-PAGE matrix onto 0.45µm polyvinylidene difluoride (PVDF) (IPVH00010, Immobilon) membrane with a semi-dry electrophoretic transfer cell (170-3940, Bio-Rad). Transfer membranes were rinsed in Tris-buffered saline with Tween 20 (TBST; 0.1% v/v Tween 20, 150 mM NaCl, 25 mM Tris-HCl, pH 7.6) and subsequently blocked in 5% w/v dry milk in TBST for one hour at room temperature. Membranes were repeatedly washed in TBST before being incubated with the primary antibody in TBST overnight at 4^{o}C or for two hours at room temperature. Horseradish peroxidase conjugated secondary antibodies against mouse and rabbit IgG (NA931V and NA934V, GE) were hybridized to the primary antibodies. The complex was detected with chemiluminescent reagent (170-5060, Bio-Rad or NEL 101, NEL102, Perkin Elmer) and visualized using a film developer (CP1000, Agfa).

Gel Staining, Tryptic Digestion and Mass Spectrometry

The SDS-PAGE matrix was stained using SimblyBlue SafeStain (LC6060, Invitrogen) or mass spec compatible silver stain (24600, Thermo). Staining was performed as described in the respective protocols. Tryptic digestion was performed using the in-gel tryptic digestion kit (89871, Thermo). Protocol was performed as described in the manual using TCEP for reduction and Iodoacetamide for alkylation.

When possible, digestion was performed inside a biosafety cabinet to reduce the prevalence of keratin contamination. Tryptic digestion was incubated at 30^{o}C overnight. The supernate was removed and placed into a clean new tube. The remaining gel pieces were subjected to a secondary extraction using 50% acetonitrile and 5% formic acid. The secondary extraction was collected and combined with the primary extraction than stored in the -20^{o}C freezer. Samples were submitted to Dr. James Clifton at Brown University EPSCoR proteomics facility. Samples were submitted as low complexity samples to be run through LC coupled MS/MS on the LTQ Orbitrap Velos mass spectrometer.

Spectral Analysis

Analysis of spectral data was performed in multiple iterations using different systems. Initial analysis was performed using the MASCOT database which uses a probability based peptide finger printing method to automatically identify and evaluate spectral data (Perkins 1999). ProteoIQ v2.2 was used to remove hits that fell below the 5% protein false discover rate threshold. ProteoIQ was used in tandem with manual curation of the raw MASCOT data to find samples that were differentially detected in the experimental and control samples. Exclusions were made based on relative abundance in the control samples, repositories of known contaminants, and subcellular location, as a result of the prerequisite fractionation (Mellacheruvu 2013). Manual curation relied heavily on the UniProt database though several cases required rigorous searches of the literature to determine protein function, possible validity, and relevance (Apweiler 2004).

CHAPTER 3

RESULTS

FANCD2 Interacting Proteins can be Detected by Mass Spectrometry.

To determine if the system was workable, several pulldowns were performed to enrich for FANCD2 immune complexes. Using a commercially available V5 antibody it was possible to enrich for both FANCD2 and monoubiquitinated FANCD2 (**Figure 1**). The remaining eluate from the immunoprecipitation did not present a differential banding pattern when stained (**Figure 2**). Lack of visual detection does not preclude detectable differences between samples, so gel pieces from the SDS-PAGE were excised and examined by mass spectrometry. The mass spectrometry results confirmed the presence of FANCD2, however it did not identify any strong candidates for further analysis (**Table 1**).

Figure 1. Immunoblot of FANCD2 immunoprecipitation

PD20 patient cells containing empty vector or corrected with FANCD2-V5-6xHis (D2-V5/6xHis) were grown to confluency and half the cells were treated with 250 nM MMC for 16 hours. Cells were harvested following treatment and then lysed in NETN300 (20 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 1 mM Na_3O_4V, 1 mM NaF, Roche protease inhibitor cocktail tablet). 1 mg of cellular extract was used for the immunoprecipitation which was supplemented with NETN100 to make a 1 mL sample. The samples were not subjected to the pre-clearing step. The commercially available V5 antibody was allowed to incubate with the sample overnight. 30 µg of cell extract was run in the first four lanes of a 3-8% Tris-Acetate gel as a control while 5 µL of the 40 µL eluate was loaded into the last four lanes to probe the success of the IP. The SDS-PAGE was performed and followed by the immunoblotting protocol. The blot was probed with the FANCD2 (NB100-182, Novus Biologicals) antibody showing that the IP was able to enrich for FANCD2 as seen in lane seven and eight.

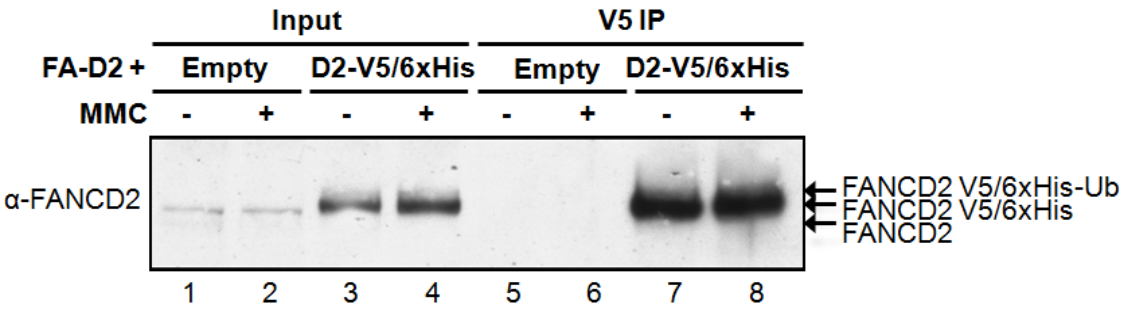

Input
V5 IP
FA-D2 +
Empty
D2-V5/6xHis
Empty
D2-V5/6xHis
MMC
- + - + - + - +
α-FANCD2
FANCD2 V5/6xHis-Ub
FANCD2 V5/6xHis
FANCD2
1 2 3 4 5 6 7 8

Figure 2. Coomassie stain of the FANCD2 immunoprecipitation

35 µL of the 40 µL of eluate described in Figure 1 were resolved by SDS-PAGE on a 3-8% Tris-Acetate gel then stained with the SimplyBlue SafeStain (LC6060, Invitrogen). The gel does not show any bands that are more prominent in the corrected line than in the hypomorphic line. Taken together with the results from the immunoblot it would suggest that the amount of differential protein present in the eluate is not above the threshold for detection using the coomassie reagent. A 1 mm wide vertical strip spanning the length of the gel was excised from lane two and lane four. The vertical strips were each separated into twelve 1 cm long pieces. These 1 cm long strips were cut into pieces no larger than 2 mm^3. Samples underwent preparation for mass spectrometry as described in the materials and methods section and were submitted for analysis by mass spectrometry. The results from lane two were used to remove any nonspecific interactions that were pulled down with the beads appearing in lane four.

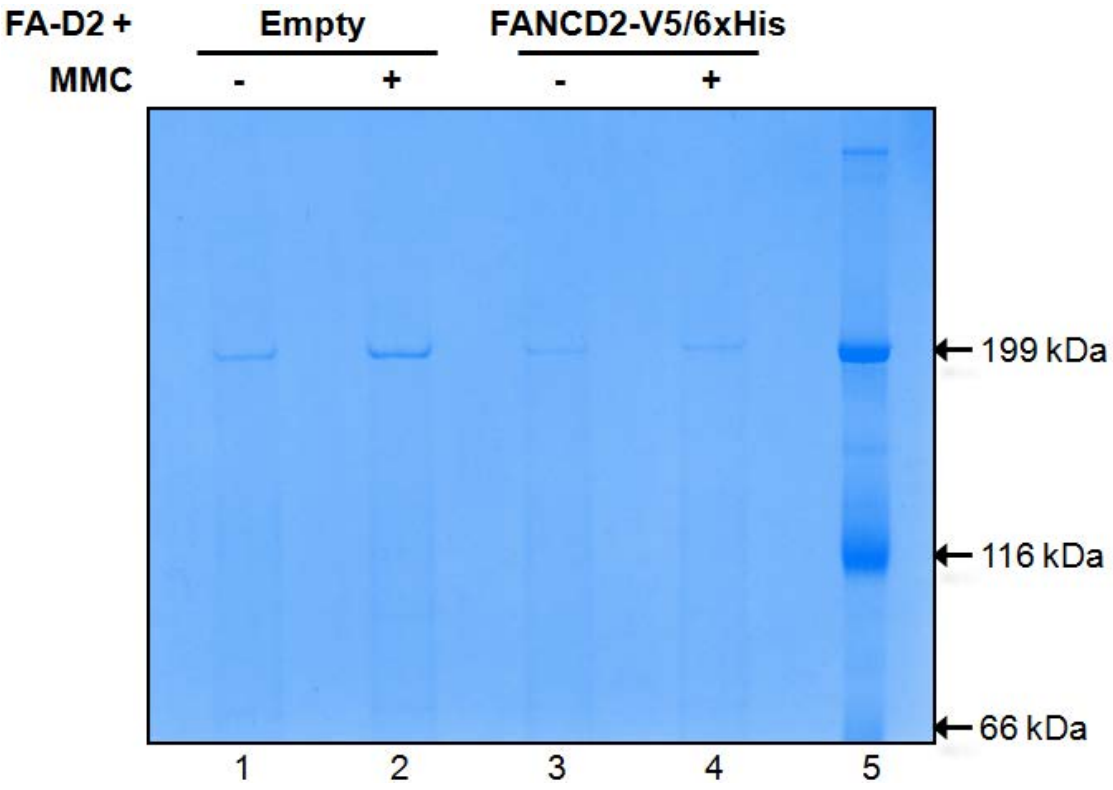

FA-D2 +
Empty
FANCD2-V5/6xHis
MMC
-
+
-
+
199 kDa
116 kDa
66 kDa
1
2
3
4
5

Table 1. Mass spectrometry results from PD20 FANCD2-V5/6xHis V5 IP

These results were analyzed using ProteoIQ version 2.2. All results were subjected to a 5% false discovery rate validation. Proteins which were detected in the empty sample were removed from the proteins that were present in the V5 IP. Only ten proteins were validated using this method. The most prominent of the detected proteins was FANCD2 confirming the success of the IP. The other proteins that were detected registered very few spectral counts. Apart from the low rate of detection for these proteins several of the proteins are cytoplasmic or secreted and therefore are not likely to be involved or affected by the chromatin targeting of FANCD2.

Protein	Function	Empty spectra	V5 spectra
BBX	HMG box transcription factor	0	1
ENO2	Maintains and protects neurons	0	1
FANCD2	Required for chromosomal stability	0	36
FLJ59433	Translation elongation factor	0	1
MYH1	Muscle contraction	0	1
RPSAP58	Ribosome constituent	0	1
S100-A14	Modulates p53 protein levels	0	1
TRIM35	Possible Tumor Suppressor	0	2
TUBA1A	Cytoskeletal protein	0	2
YBX1	Regulation of transcription	0	1

pLenti 6.2 FANCD2-V5 is Functionally Incorporated into the Cell

Using the pLenti FANCD2-V5 vector to correct the cells shows a functional incorporation of the protein into the cellular machinery. The FANCD2-V5 undergoes ubiquitination following exposure to MMC (**Figure 3**) and is also localized to chromatin (**Figure 4**). To adequately correct the sensitivity to crosslinking agents FANCD2 must undergo monoubiquitination, chromatin localization, and discrete nuclear foci formation. Monoubiquitination demonstrates that the protein is at least interacting with the components of the core complex which ubiquitinate the protein. Chromatin localization places the protein in the context of DNA repair and in proximity with other DNA repair proteins. The ability to enrich activated chromatin associated FANCD2 allows for specific screening of interaction occurring in the context of an active DNA repair environment. The incorporated protein can be targeted by the commercial V5 antibody for enrichment, and should function as a useful platform to detect proteins that interact with activated FANCD2 in the context of DNA repair.

Figure 3. pLenti 6.2 FANCD2-V5 is efficiently ubiquitinated following exposure to crosslinking agents

PD20 cells stably corrected with pLenti 6.2 FANCD2-V5 and a control line with stably incorporated pLenti 6.2 LacZ-V5 were grown to confluency and half of the cells were treated with 250 nM MMC for 16 hours. The cells were harvested and lysed in 2% SDS lysis buffer. The whole cell lysate was quantified using the BCA assay and samples were normalized to 30 μg than resolved by SDS-PAGE followed by immunoblotting with a V5 antibody. The immunoblot clearly shows an induction of monoubiquitinated FANCD2 following MMC treatment in the corrected line. This indicates that a sizeable portion of the FANCD2 that is expressed is functionally incorporated into the cell.

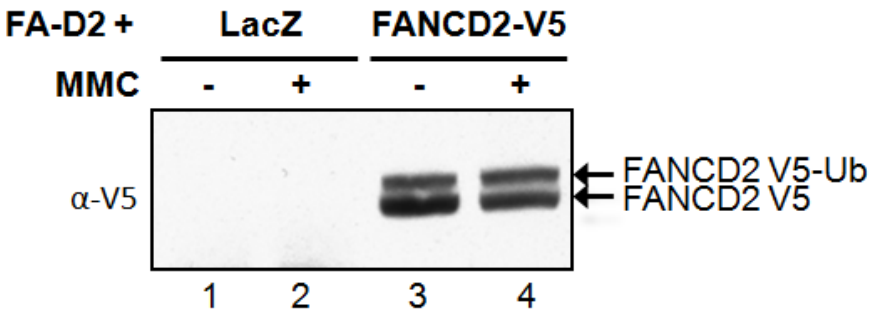
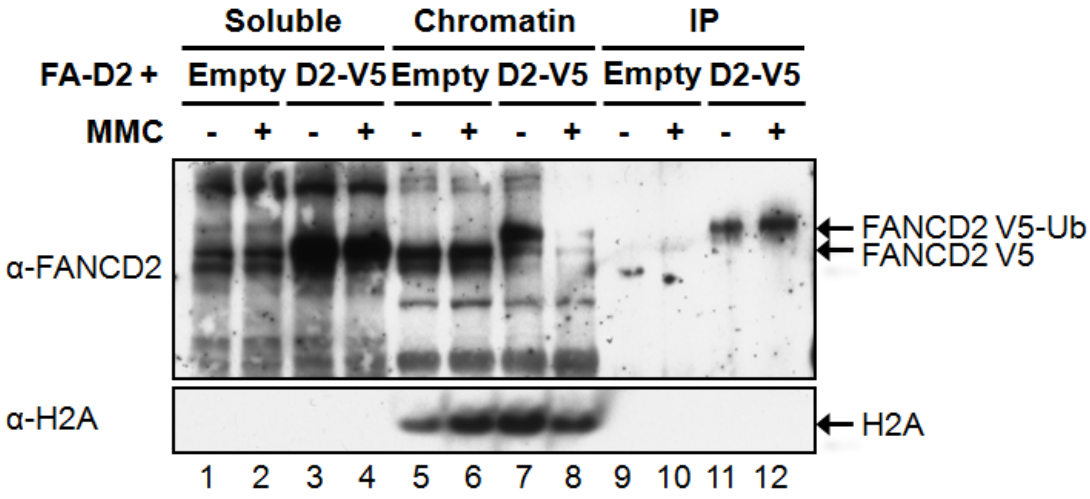

FA-D2 +
LacZ
FANCD2-V5
MMC
-
+
-
+
α-V5
FANCD2 V5-Ub
FANCD2 V5
1
2
3
4

Figure 4. pLenti 6.2 FANCD2-V5 is localized to chromatin following exposure to crosslinking agents

PD20 cells stably corrected with pLenti 6.2 FANCD2-V5 and a control line with stably incorporated pLenti 6.2 LacZ-V5 were grown to confluency and half of the cells were treated with 250 nM MMC for 16 hours. The cells were fractionated as described using the CSK buffer and NRB with micrococcal nuclease. The IP was performed using a V5 antibody with the chromatin associated fraction. Immunoblotting with FANCD2 shows the effect of enrichment by the fractionation and the immunoprecipitation. Lanes one through four show the soluable fraction which has a large proportion of the nonubiquitinated FANCD2. Lanes five through eight show the Chromatin associated fraction which as a clear enrichment for monoubiquitinated FANCD2. The nonubiquitinated form is able to associate with chromatin but at a much lower frequency possibly suggesting that monoubiquitination stabilizes the association. Lanes nine through twelve show that the IP is able to pull down the monoubiquitinated FANCD2 from the chromatin associated fraction. The H2A immunoblot demonstrates that the fractionation was specific for chromatin associated proteins. Histones are normally very tightly bound to DNA in the nucleosome, as expected H2A is absent from the soluble fraction but present in the chromatin associated fraction.


Soluble
Chromatin
IP
FA-D2 +
Empty
D2-V5
Empty
D2-V5
Empty
D2-V5
MMC
- + - + - + - + - + - +
α-FANCD2
FANCD2 V5-Ub
FANCD2 V5
α-H2A
H2A
1 2 3 4 5 6 7 8 9 10 11 12

Fractionation Allows Isolation of FANCD2 Immune Complex Members from Endogenous Systems

Isolating the chromatin associated portion of cells has shown to enrich activated FANCD2. However the fractionation method required large volumes of cells to generate large enough protein volumes to successfully IP from. Using transformed cells such as HeLa cells allows for the rapid generation of large volumes of high protein content cells. Using unmodified HeLa cells removes the option to pull down using the V5 epitope tag. The cells were fractionated and then underwent a FANCD2 IP using an antibody against FANCD2 to enrich for FANCD2 immune complexes (**Figure 5**). The eluate was resolved by SDS-PAGE and stained to observe differential banding patterns which could be excised and submitted for analysis by mass spectrometry (**Figure 6**). The analysis revealed a number of functionally diverse candidates with varying detection strengths (**Table 2**). One of the difficulties with the endogenous system is the lack of specificity with the antibody used. The best available antibody for FANCD2 has multiple targets within the cell. The issue of nonspecific binding is exacerbated by the PIS control which also binds several proteins nonspecifically and may invalidate or mask interacting partners. While the HeLa system is able to identify several candidates the large amount of contaminants and nonspecific interactions in the data demonstrate the need for a more sensitive system to screen for interacting proteins.

Figure 5. Enrichment of FANCD2 from HeLa cells using FANCD2 immunoprecipitation

HeLa cells were grown to confluency and half were treated with 250 nM MMC for sixteen hours. The cells were fractionated using CSK buffer and NRB supplemented with micrococcal nuclease. 1 mg of the chromatin associated fraction was incubated with a FANCD2 antibody (NB100-182, Novus Biologicals) and pulled down using magnetic beads. The sample was also incubated with rabbit pre immune serum and magnetic beads as well as just the magnetic beads. Only the MMC treated The IPs are shown in the blot above as they were the only samples to be submitted to proteomic analysis. The immunoblot for FANCD2 (sc-20022, Santa Cruz) demonstrates that monoubiquitinated chromatin associated FANCD2 is present in the FANCD2 IP and is not pulled down nonspecifically by rabbit serum or the magnetic beads. The Immunoblot for H4 shows that the chromatin fractionation was successful.

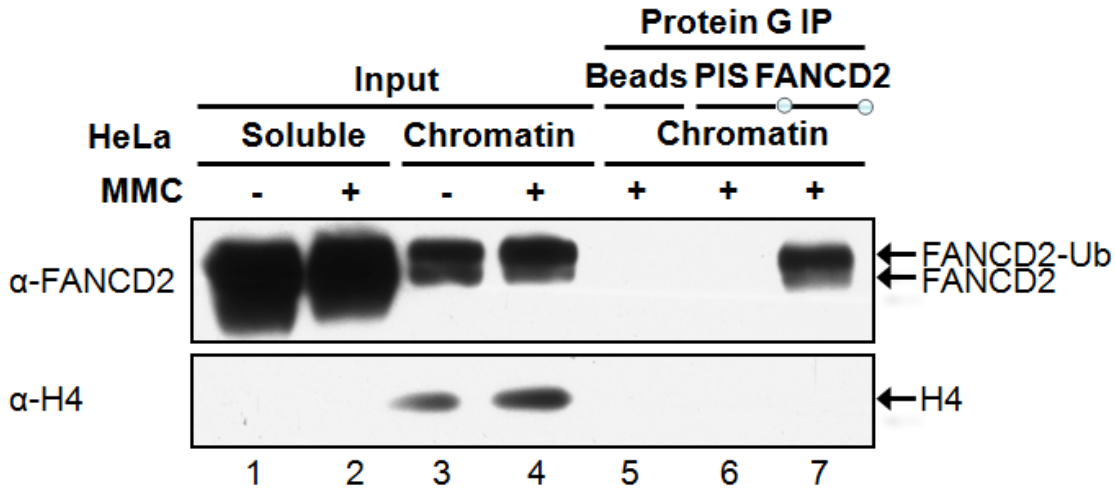

Protein G IP
Input
Beads
PIS FANCD2
HeLa
Soluble
Chromatin
Chromatin
MMC
- + - + + + +
α-FANCD2
FANCD2-Ub
FANCD2
α-H4
H4
1 2 3 4 5 6 7

Figure 6. Silver stain of FANCD2 immune complexes from HeLa cell chromatin fractions

The remaining eluates from the IP in **Figure 5** were resolved by SDS-PAGE then were stained with the mass spectrometry compatible silver stain kit. The gel contains the eluates of the bead pull down, the pre immune serum (PIS) pull down, and the FANCD2 pull down. The silver stain shows nine differentially staining regions in the gel between the PIS and FANCD2 pull downs. The nine regions indicated were excised from both lanes of the gel so that nonspecific interaction could be removed from the FANCD2 IP results. The bands were excised as fragments approximately 1 mm tall and 1 cm wide which were subsequently cut into approximately 1 mm^3 cubes. The bands were distained were than subjected to the tryptic in gel digestion protocol. The six most prominent bands were submitted for mass spectrometry analysis.

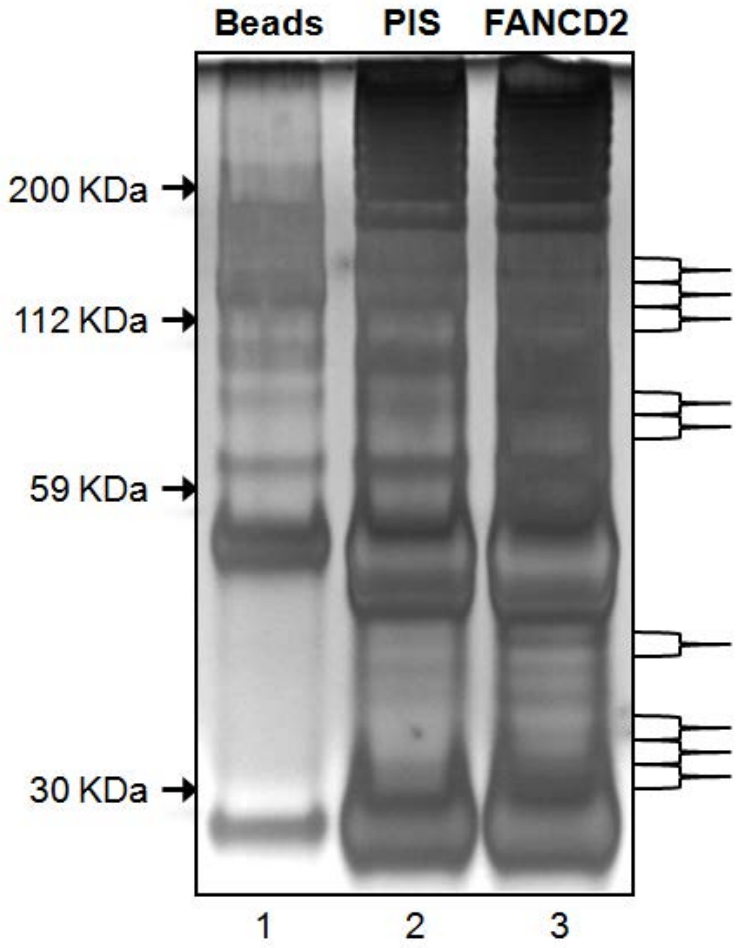

Beads
PIS
FANCD2
200 KDa
112 KDa
59 KDa
30 KDa
1
2
3

Table 2. Abridged list of candidates identified from HeLa FANCD2 immune complexes

These results were analyzed using ProteoIQ version 2.2. All results were subjected to a 5% false discovery rate validation. Proteins which were detected in the pre immune serum sample were removed from the proteins that were present in the FANCD2 IP. Using the Uniprot database Proteins that were detected that are found solely in the cytoplasm or extra cellular matrix of the cell were deemed as nonspecific because of the prefractionation before the IP which should have removed these interactions (Apweiler 2004). While working in a biosaftey cabinet and taking care to wipe all surfaces and instruments down with ultrapure water, it is still not possible to remove all environmental contamination. Several candidates were identified with this screen and not all positive results are displayed on this list. While several candidates have a significant number of spectral counts there are still many which have very few spectral counts. Candidates detected hail from a diverse functional background.

Protein	Function	Empty spectra	V5 spectra
ADAR	RNA processing	0	5
AZI1	Centrosomal protein	0	10
CCAR1	Functions as a p53 coactivator	0	4
CEP135	Involved in centriole biogenesis	0	4
DHX29	ATP-binding RNA helicase	0	1
DHX30	Interacts with DNA and SSBP1	0	20
DHX9	Unwinds DNA and RNA	0	32
FANCD2	Required for chromosomal stability	0	1
LRPPRC	Binds to poly(A) mRNAs	0	2
MYBBP1A	May activate or repress transcription	0	4
NUP155	Component of nuclear pore complex	0	4
NUP160	Involved in poly(A)+ RNA transport	0	2
RBM6	Binds poly(G) RNA homopolymers	0	3
SF3B1	Identified in spliceosome C complex	0	15
SIN3B	Acts as a transcriptional repressor	0	2
SMC3	Required for chromosome cohesion	0	11
STAG1	Component of cohesin complex	0	2
SUGP2	May play a role in mRNA splicing	0	3
TP63	transcriptional activator or repressor	0	3

Large Scale Enrichment of FANCD2 Immune Complexes from PD20 Patient Cells Identifies Several Interacting Candidates

Previous work with the PD20 patient cells showed it was difficult to generate a large enough volume of the chromatin associated fraction to be able to enrich enough FANCD2 immune complexes to detect by silver stain. Large volumes of cells were cultured to perform fractionations and subsequent IPs on. The process was shown to enrich for chromatin associated monoubiquitinated FANCD2 (**Figure 7**). The silver stain showed sixteen detectable differential bands or regions between the IP samples which were analyzed by mass spectrometry (**Figure 8**). The mass spectrometry analysis revealed several candidate proteins, which have been broken down into six different groups which includes nucleosome remodeling, nuclear matrix, DNA repair, transcription regulation, chromosome maintenance, and a miscellaneous proteins group (**Table 3-8**). All of these tables were generated using ProteoIQ 2.2 analysis. All results were subjected to a 5% false discovery rate validation. Proteins that were detected that are found solely in the cytoplasm or extra cellular matrix of the cell were deemed as nonspecific because of the prefractionation before the IP which should have removed these interactions. Common contaminants of this method were detected and removed from candidate lists. Immune complex candidate proteins which were detected at an equal or higher rate in the sample lacking any V5 epitope were removed from the list and seen as a nonspecific interaction.

Figure 7. Enrichment of chromatin associated monoubiquitinated FANCD2 from PD20 patient cells

PD20 empty and PD20 pLenti 6.2 FANCD2-V5 cells were each plated into sixty four 15 cm dishes and grown to confluency. All of the cells were treated with 250 nM MMC for 16 hours and subsequently harvested. The cell pellets were fractionated using the CSK buffer to extract the soluable fraction (S) and ATM lysis buffer with benzonase to extract the chromatin associated fraction (**C**). 5.2 mg of the chromatin fraction was incubated with the V5 agarose beads to pull down FANCD2-V5 immune complexes. The complexes were resolved by SDS-PAGE alongside 10 µg amounts of the cellular fractions to be used as controls. Immunoblotting with H3 demonstrates the success of the fractionation and the FANCD2 immunoblot shows that FANCD2 was pulled down during the immunoprecipitation.

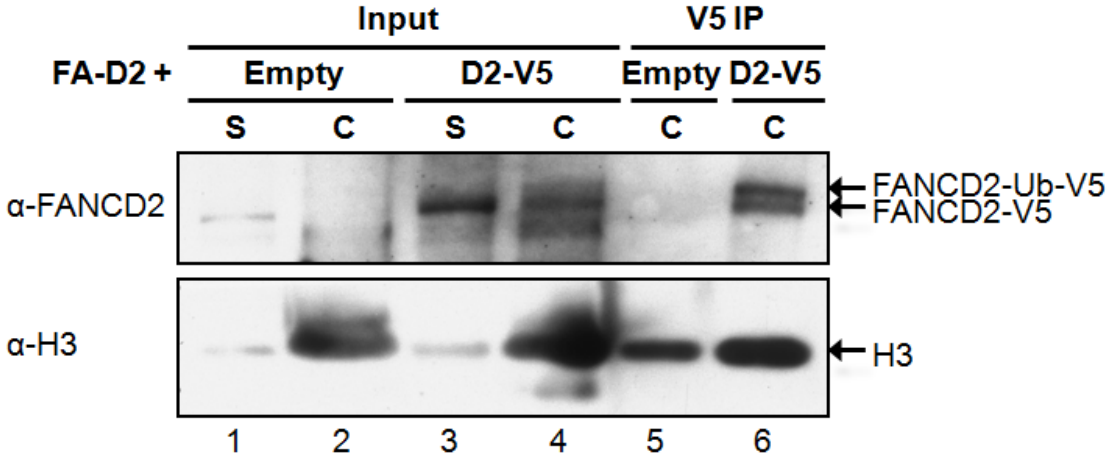

Input
V5 IP
FA-D2 +
Empty
D2-V5
Empty
D2-V5
S
C
S
C
C
C
α-FANCD2
FANCD2-Ub-V5
FANCD2-V5
α-H3
H3
1
2
3
4
5
6

Figure 8. Silver stain of FANCD2 immune complexes from the PD20 cell chromatin fraction

The remaining eluates from the IP in **Figure 7** were resolved by SDS-PAGE than was stained with the mass spectrometry compatible silver stain kit. The gel shows two lanes of 10 µgs of the raw chromatin associated fraction in addition to the two V5 IP eluates. The silver stain shows sixteen differentially staining regions in the gel between the PD20 empty and the PD20 FANCD2-V5 pull downs. The sixteen regions indicated were excised from both lanes of the gel so that nonspecific interaction could be removed from the FANCD2 IP results. The bands were excised as fragments approximately 1 mm tall and 1 cm wide which were subsequently cut into approximately 1 mm^3 cubes. The bands were distained then subjected to the tryptic in gel digestion protocol. It should be noted that bands do not necessarily indicate the presence of a single discrete protein but are simply an abundance of protein above the threshold for detection. Several different protein co-migrating may contribute to the overall concentration of proteins possibly causing large smeared bands or regions with various detectable proteins. To reduce the problem of protein contamination work was performed in a biosaftey cabinet and care was taken to wipe all surfaces and instruments down with ultrapure water. The gel was also wrapped in saran wrap to prevent exposure to additional sources of contamination while it was being imaged, resulting in several observable distortions in the captured image of the gel.

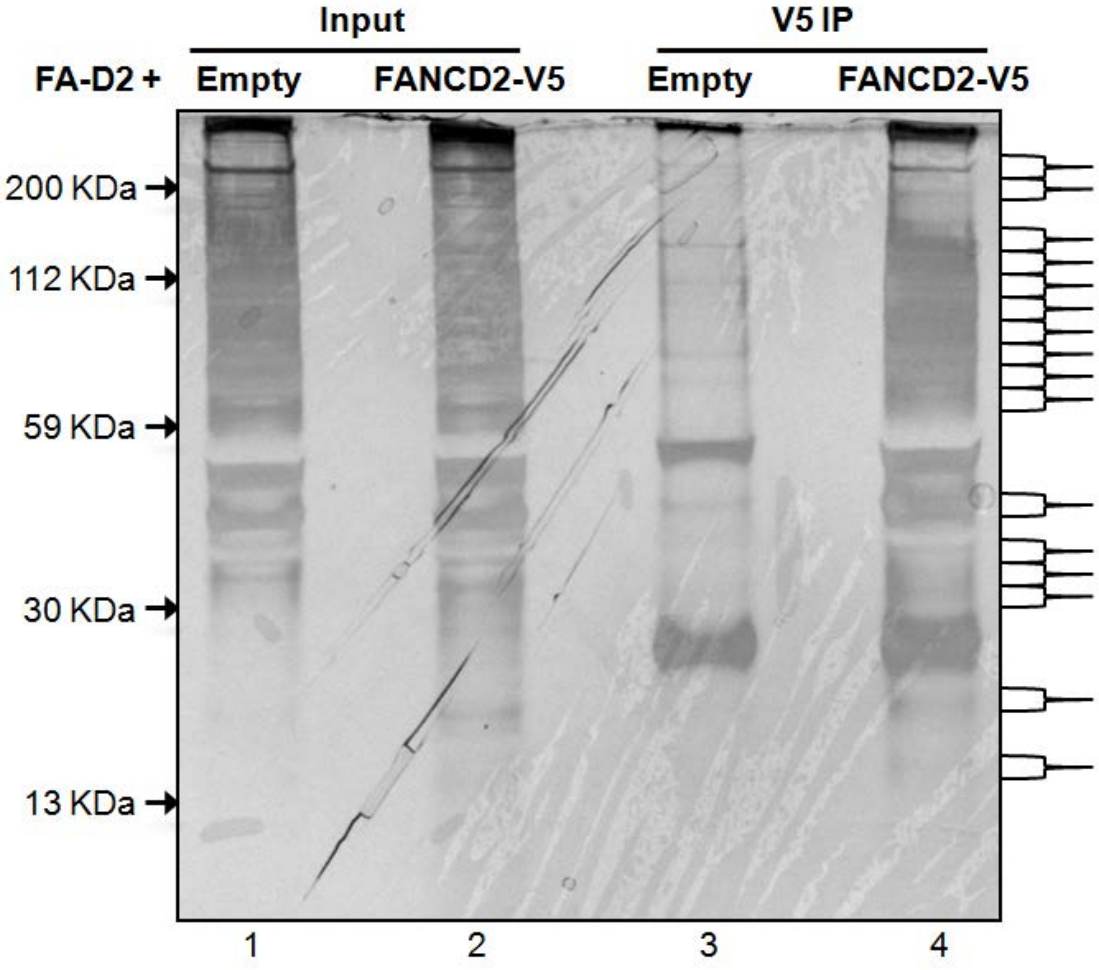

Input
V5 IP
FA-D2 +
Empty
FANCD2-V5
Empty
FANCD2-V5
200 KDa
112 KDa
59 KDa
30 KDa
13 KDa
1
2
3
4

Table 3. Nucleosome remodeling proteins in FANCD2-V5 immune complexes

This table shows the candidates that were identified as nucleosome remodeling components. Numerous remodeling complex members were identified including the ASAP, BAF, FACT, and NuRD complexes. Also grouped in with this category are the histone proteins that were detected. It is difficult to identify the histone variants with the methods that were used in this screen so the variants represented in the table are reflection of the inherent uncertainty in different isoforms and postranslation modifications. More detailed analysis of histone members would require special proteomic techniques and parameters.

Protein	Function	Empty spectra	V5 spectra
ACIN1	Component of ASAP complex apoptosis and splicing complex	1	3
ANP32B	Cell cycling nucleasome chaperone	1	2
BAG6	Chaperone involved in p53 activity and chromatin regulation H3K4me2	0	2
BRD3	Binds actlyated histones	0	1
CDC73	Transcription regulation nucleosome remodeling complex H3 methylation	0	5
FBL	Histone methyl transferase H2AQ104me	3	8
H1F0	Histone H1	1	6
H1FX	Histone H1	2	12
H2AFJ	Histone H2A	1	13
HIST1H1B	Histone H1	11	18
HIST1H1C	Histone H1	18	73
HIST1H1D	Histone H1	19	72
HIST1H2AD	Histone H2A	1	12
HIST1H2BK	Histone H2B	11	46
HIST1H2BL	Histone H2B	13	46
HIST1H2BN	Histone H2B	13	46
HIST1H4H	Histone H4	6	19
HIST2H3A	Histone H3	3	12
KIF11	Component of large chromatin remodeling complex	0	5
NAT10	Able to actylate histones	0	12
PHB2	HDAC recruitment	3	9
RELA	Transcriptional regulator with DDX1 interacts with HDACs	0	4
SAP18	Nucleosome remodeling transcription	3	7
SMARCA4	Component of the BAF chromatin remodeling complex	1	3
SMARCA5	Helicase with nucleosome remodeling activity	2	9
SMARCB1	Chromatin remodeling	1	2
SMARCC2	Chromatin remodeling for transcriptional activation/repression	5	13
SNF2L1	Nucleosome remodeling factor	1	5
SUPT16H	Nucleosome reorganizer	1	14
TRIM28	Recruits NuRD degrades p53	2	6

Table 4. Nuclear matrix proteins in FANCD2-V5 immune complexes

The nuclear matrix proteins identified largely fall into two groups relating to the nuclear envelope. The Lamin proteins were detected with high spectral counts but were also somewhat abundant in the control sample, though there is a clear increase in the number of spectra detected in the V5 sample. The other large group relates to the nuclear pore complex. Several nuclear pore proteins were identified in the screen and in past screens which has been previously published on (Boisvert 2013).

Protein	Function	Empty spectra	V5 spectra
FNBP3	Nuclear matrix	1	11
LMNA	Nuclear membrane chromatin interacting	38	101
LMNB1	Nuclear membrane chromatin interacting	25	51
LMNB2	Nuclear membrane chromatin interacting	23	52
NUP107	Nuclear pore complex	0	5
NUP188	Component of nuclear pore complex	0	2
NUP93	Nuclear pore complex	5	11
ODF2	Centrosome matrix required for localizing PLK1	0	4
RANBP2	E3 for SUMO of UBE2I component of nuclear pore complex required for export	1	3

Table 5. DNA repair proteins in FANCD2-V5 immune complexes

DNA repair proteins identified in the screen are listed in the table above. While many of the proteins in this list are familiar to the FA pathway very few have a highly enrich spectral count in the V5 sample. While it is welcoming to see known FANCD2 interacting proteins such as PCNA in the screen the strongest candidates identified in this group are VCP and SFPQ (Howlett 2009). These proteins may have a direct role in recruiting FANCD2 to sites of DNA damage.

Protein	Function	Empty spectra	V5 spectra
ASCC3	3'-5' DNA helicase involved in repair of alkylated DNA	0	3
DDX1	RNA clearance at DNA DSB	1	2
ILF2	Stabilizes Ku70/Ku80	0	6
PARP1	Base excision repair chromatin architecture required for PARP9 and 53BP1 recruitment	0	5
PCNA	TLS	0	1
PSMD1	Regulatory subunit of proteasome	0	3
REV1	DNA repair	0	1
SFPQ	Nucleotide binding may be involved in ss invasion and NHEJ	1	9
VCP	Recruited to stalled replication forks by SPRTN Recruits 53BP1	1	13

Table 6. Transcription regulation proteins in FANCD2-V5 immune complexes

A large number of proteins involved in the regulation of transcription were identified. These proteins are involved in both transcriptional activation and repression. With FANCD2 being implicated in transcriptional regulation it makes sense that it would interact with other transcription proteins (Park 2013). Many of the transcription factors identified hail from different functional backgrounds and do not readily divide into groups or complexes.

Protein	Function	Empty spectra	V5 spectra
AIMP2	Prevents p53 degredation	1	4
ALYREF	mRNA transport THO complex component	0	5
ATAD2	Transcriptional coactivator of ESR1	1	3
BTF3	Transcription	2	4
CHCHD3	BAG1 repressor	0	1
CSNK2A1	Cell signaling apoptosis transcription	0	2
CTNNB1	Negative regulator of chromosome cohesion	0	15
CTNND1	Catennin Wnt signaling	1	23
DDX17	Helicase transcriptional regulation	10	34
DDX3X	Transcription regulation	7	19
DDX3Y	ATP dependant RNA helicase	5	14
DDX5	Transcritiptional coactivator p53 RUNX2	15	45
FHL2	Transcription regulation	0	6
FLII	May be a transcriptional coactivator associated with hormone activated nuclear receptors	1	9
GAPDH	Carbohydrate degredation apoptosis	8	22
HELZ2	Interacts with and coactivates PPARA and PPARG	0	7
HMGA1	Transcription regulation	0	2
HMGN4	chromatin	0	1
HNRPK	Interacts with p53 and p21 necessary for apoptosis	1	2
MYBBP1A	Transcriptional activator or repressor	2	7
NF2	Tumor supression	0	2
PELP1	Coactivator of ER transcription corepressor of other hormone receptor transcription	0	1
RPB1	DNA dependant RNA polymerase component of RNA pol II	0	7
RPB2	Component of RNA pol II	2	10
SAFB2	Binds matrix attachment region DNA estrogen receptor corepressor	1	8
SND1	Transcriptional and p100 coactivator	0	7
SNW1	Transcriptional regulation and mRNA splicing p53 interacting	2	5
STAU1	DNA binding	1	3

Table 7. Chromosome maintenance proteins in FANCD2-V5 immune complexes

A large amount of proteins involved in chromosomal maintenance were identified in the screen. Multiple members of many groups were identified such as, centrosomal proteins, spindle proteins, replication initiation, cohesin, and condensin. Two of the MCM proteins identified in the screen have recently been shown to interact with FANCD2 (Lossaint 2013). Nearly all of the SMC proteins were identified in the screen which implicates FANCD2 interacting with cohesin, condensin, and the SMC5-SMC6 complex. The large number of centrosomal proteins detected in the screen also suggests an interaction between FANCD2 and the centrosome.

Protein	Function	Empty spectra	V5 spectra
AKAP8L	Chromatin condensation	1	6
AURKB	Regulates chromosome alignment and stability as well as p53	1	2
AZI1	Centrosome	0	3
CDC42	Apoptosis transcription Spindle	0	2
CDK1	Regulates cell and centrosome cycling	2	7
CEP135	Centrosomal protein centriole biogenesis	0	2
CEP250	Important role in centrosome cohesion	0	3
CKAP2	P53 dependant cell cycle regulator	0	3
DYNC1LI1	Chromosome migration	0	2
HAUS3	Centrosome integrity	1	2
HAUS6	Spindle assembly interacts with PLK1	0	3
LZTS2	Centrosome and Wnt signaling	0	1
MCM5	DNA replication initiation	0	4
MCM7	DNA replication initiation	0	4
NCAPD2	Component of condensin I complex	0	6
NDC80	Chromosome segregation	0	1
NEK7	Centrosome spindle formation	0	2
NUDCD2	Centrosome	0	1
NUMA1	Involved in teathering centrosome to tubulin and spindle assembly	12	31
PCM1	Required for centrosome assembly and function interacts with CEP 250	2	14
PDS5	Regulator of sister chromatid cohesion during mitosis	0	7
PPP1CA	Chromatin structure	1	4
PPP1CB	Regulates chromatin structure	0	3
PPP2CB	Centrosome spindle	0	2
PPP2R1A	Chromosome segregation	0	4
SLC25A5	Chromosome segregation	1	26
SMC1A	Involved in chromosome cohesion during cell cycle and damage BRCA1 interactor	0	19
SMC2	Component of condensing complex	2	12
SMC3	Required for chromosome cohesion	4	24
SMC4	Component of condensin complex	1	12
SMC6	Involved in dsDNA break HRR	0	5
SPAG5	Required for normal chromosome segregation	0	3
TNKS1BP	Binds TNKS1 and TNKS2 colocalizes with chromosomes during mitosis	0	3
TOP1	Cuts one strand of DNA during replication and transcription	1	9
TOP2A	DNA topoisomerase 2 Makes DNA ds breaks required for mitotic segregation	0	10

Table 8. Other candidate proteins in FANCD2-V5 immune complexes

The remaining proteins identified in the screen are mostly involved in nuclear import and ubiquitination. Several nuclear import proteins were identified however, it is unclear how these proteins interact with chromatin associated FANCD2. Ubiquitin was strongly enriched in the V5 sample however this ubiquitin could be a result of the monoubiquitination of FANCD2. With the experimental design the program has no way of determining the origin of ubiquitin in the sample and it is highly possible that the ubiquitin was cleaved off of FANCD2 or any of the other ubiquitinated proteins in the pathway. The analysis did not include searching for diglycine modifications indicating protein ubiquitination, so it is also unknown which proteins in the sample were ubiquitinated.

Protein	Function	Empty spectra	V5 spectra
IPO5	Nuclear import	0	3
IPO9	Nuclear protein import	0	2
KPNB1	Nuclear import	1	8
TNPO1	Nuclear transport histones	1	8
UBC	Ubiquitin	9	82
USP5	Degrades K48 ubiquitin linkages causes accumulation of p53 (Dayal 2009)	0	5

Validating Structural Maintenance of Chromosomes Candidates

One of the strongest groups of candidates from the mass spectrometry screen was the structural maintenance of chromosomes proteins. SMC1A, SMC2, SMC3, SMC4 and SMC6 were detected in the large scale patient cell IP (**Table 7**). SMC3 was also detected in the endogenous IP system (**Table 2**). SMC1 and SMC3 are component of the cohesin complex along with STAG1 which was detected in the endogenous system (**Table 2**). The cohesin complex sister chromatids together which allows for the identification of sister chromatids (Rudra 2013). SMC2 and SMC4 are members of the condensing complex. CAPD2 is also a member of condensin which was detected in the PD20 patient screen (**Table 7**). The condensin complex is required for the proper condensation and segregation of chromosomes (Hirano 2012). The SMC5-SMC6 complex plays a role in DNA damage repair though this may be mediated through cohesin recruitment (Potts 2006). Components of these complexes were detected in the FANCD2 immune complexes by immunoblotting (**Figure 9**). The input in **Figure 9** shows that there may be less SMC protein expressed in FANCD2 hypomorphic cells following exposure to MMC, however expression of these proteins does not apper to be largely affected by FANCD2 or MMC (**Figure 10**). The dynamic of the interaction between FANCD2 and these proteins require further characterization. Because these proteins are involved in genomic stability it is possible that these proteins may have some overlapping functions with maintaining genomic stability. Using MAGI to query the cancer genome atlas shows that mutations in both the cohesin (5.96%) and condensin (5.42%) networks are common in cancer.

Figure 9. Structural maintenance of chromosomes proteins are present in FANCD2 immune complexes

PD20 empty and PD20 pLenti 6.2 FANCD2-V5 cells were treated with 250 nM MMC for 16 hours and subsequently harvested. The cell pellets were fractionated using the CSK buffer to extract the soluable fraction and ATM lysis buffer with benzonase to extract the chromatin associated fraction. The chromatin fraction was incubated with the V5 agarose beads to pull down FANCD2-V5 immune complexes. The complexes were resolved by SDS-PAGE alongside 10 µg amounts of the chromatin fractions to be used as controls. Immunoblotting with the antibodies shown demonstrates that SMC1A, SMC4, SMC6, CAPD2 and CAPD3 are all present in FANCD2 immune complexes. These proteins represent cohesin, condensin-I, condensin-II, and the SMC5-SMC6 complex.

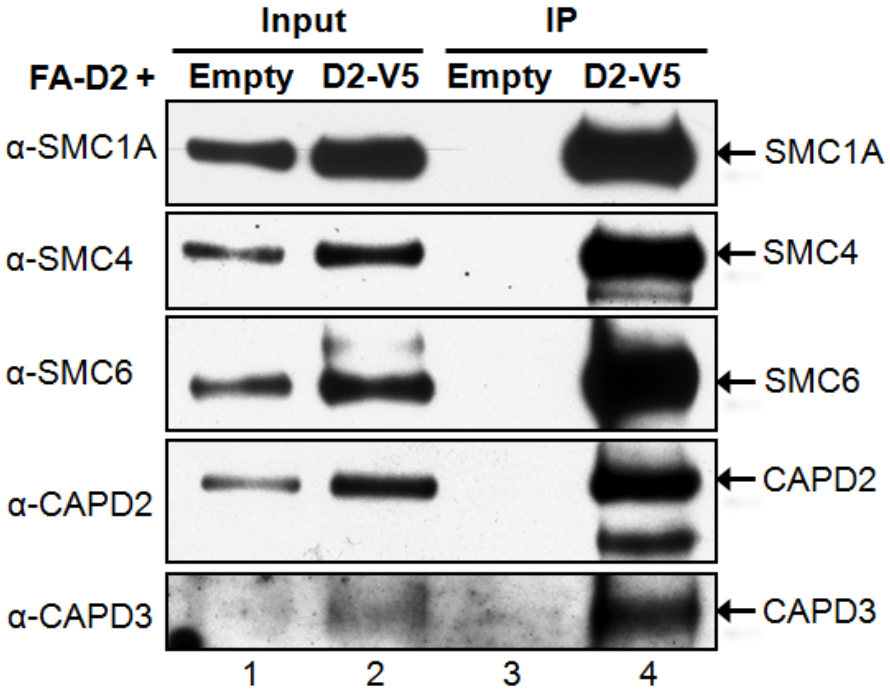
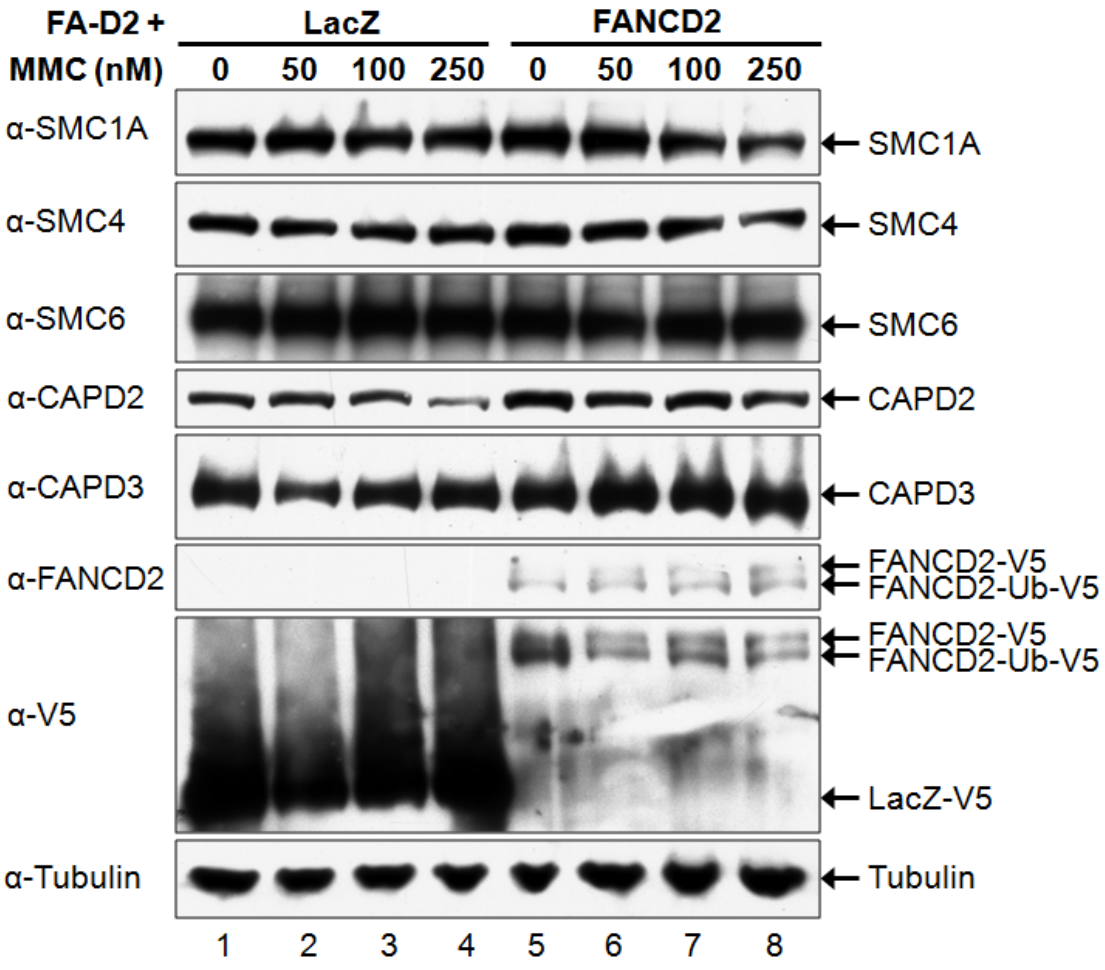

Input
IP
FA-D2 +
Empty
D2-V5
Empty
D2-V5
α-SMC1A
SMC1A
α-SMC4
SMC4
α-SMC6
SMC6
α-CAPD2
CAPD2
α-CAPD3
CAPD3
1
2
3
4

Figure 10. Structural maintenance of chromosomes proteins expression in PD20 patient cells

PD20 pLenti 6.2 LacZ-V5 and PD20 pLenti 6.2 FANCD2-V5 cells were treated with different doses of MMC (0nM, 50nM, 100nM, 250nM) for 16 hours and subsequently harvested. The cell pellets were lysed in 2% SDS lysis buffer to survey total levels of cellular expression. 30 μg of the lysate was loaded into a gel and then resolved by SDS-PAGE. Immunoblotting with the antibodies shown demonstrates that SMC1A, SMC4, SMC6, CAPD2 and CAPD3 are all present in both corrected cells and cells with hypomorphic FANCD2 expression. The profile also shows that the proteins are not grossly affected by the presence of MMC within the cell. This result excludes the possibility that the association is caused by increased expression of structural maintenance of chromosomes proteins within the cells following MMC exposure.

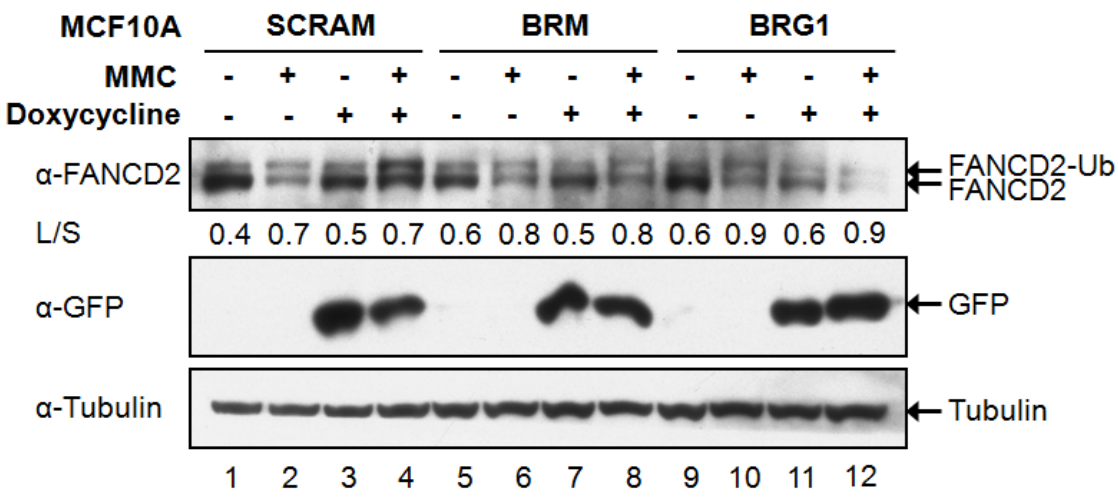

FA-D2 +
LacZ
FANCD2
MMC (nM)
0
50
100
250
0
50
100
250
α-SMC1A
SMC1A
α-SMC4
SMC4
α-SMC6
SMC6
α-CAPD2
CAPD2
α-CAPD3
CAPD3
α-FANCD2
FANCD2-V5
FANCD2-Ub-V5
α-V5
FANCD2-V5
FANCD2-Ub-V5
LacZ-V5
α-Tubulin
Tubulin
1
2
3
4
5
6
7
8

FANCD2 may Interact with Components of the SWI/SNF Complex

Several SWI/SNF proteins were also identified in the mass spectrometry screen. SMARCA4, SMARCA5, SMARCB1, and SMARCC2 were all identified in the patient cells as members of FANCD2 immune complexes along with several other nucleosome remodeling proteins (**Table 3**). Among the other proteins discovered was SUPT16H which is known to interact with SWI/SNF proteins in complexes (**Table 3**). The SWI/SNF proteins are important for transcriptional regulation, determination of cellular fate, and tumor suppression (Lu 2013). Using MAGI to query the cancer genome atlas shows that mutations in the SWI/SNF network are very common in cancer (16.14%). Using inducible knockdown cells for SWI/SNF components it was demonstrated that FANCD2 expression is affected by knockdown of SWI/SNF components (**Figure 11**). The relationship that SWI/SNF proteins have with FANCD2 may be more related to the canonical function of SWI/SNF proteins as chromatin remodelers and transcription regulators. It is possible that the interaction between FANCD2 and SWI/SNF proteins may be upstream of transcription or it may be involved in stabilizing FANCD2 protein levels.

Figure 11. SWI/SNF knockdown does not affect FANCD2 monoubiquitination

The MCF10A cells used have a stably integrated cassette, which expresses a shRNA and GFP in the presence of doxycycline. The different cells have different knockdown targets, SMARCA2/BRM, SMARCA4/BRG1, and a scrambled shRNA which has no target. The cells were treated with 0.01 µg/mL of doxycycline for 72 hours. During the final 24 hours of doxycycline treatment the cells were co-treated with 200 nM MMC. The cells were harvested and lysed with 2% SDS lysis buffer to extract the proteins from the cells. 30 µg of cell lysate was loaded into gels to be resolved by SDS-PAGE and subsequent immunoblotting. The cell were probed for GFP to assess if the shRNA was expressed, GFP expression in this case is a proxy for knockdown. The samples were also probed for FANCD2 to determine if knockdown of SWI/SNF component would impede the ubiquitination and therefore activation of the FA pathway. In the MCF10A cells used FANCD2 undergoes a normal ubiquitination response to MMC both with and without doxycycline mediated knockdown.


MCF10A
SCRAM
BRM
BRG1
MMC
Doxycycline
α-FANCD2
FANCD2-Ub
FANCD2
L/S
0.4 0.7 0.5 0.7 0.6 0.8 0.5 0.8 0.6 0.9 0.6 0.9
α-GFP
GFP
α-Tubulin
Tubulin
1 2 3 4 5 6 7 8 9 10 11 12

CHAPTER 4

DISCUSSION

Identifying FANCD2 Interacting Candidate Proteins

Using mass spectrometry to identify novel interacting partners has been a challenging and rewarding technique. There are many difficulties in working with a large low abundance protein, but the data generated from the mass spectrometry screen has shown many new interesting results. The technique was also able to detect some already known interactions such as PCNA (**Table 5**, Howlett 2009) and the MCM proteins (**Table 7**, Lossaint 2013). Developing a method which is able to pull down chromatin associated FANCD2 immune complexes to detect by mass spectrometry has opened the door for similar IP based experiments.

The screen has identified several candidates from diverse functional groups which may indicate a larger functional role for FANCD2 and the FA-BRCA pathway. The numerous strong candidates identified by this method will require subsequent validation and characterization. This process has been started on several proteins however the large volume of data will require a systematic and methodical approach to utilize the data generated from the screen. The unbiased nature of the screen also does not discriminate against upstream or downstream proteins and does not occlude subtle events. Upstream events can be easily assayed by looking at FACND2 monoubiquitination, chromatin localization, and nuclear foci formation, in response to interstrand crosslinking agents which are all well developed assays within the lab.

Genomic instability can be assayed through metaphase spreads to determine if a protein interaction is involved in promoting repair downstream of FANCD2. But the need for new methods may be most realized while investigating subtle effects, which may only be viewable during specific conditions, or may require more information from experiments than is currently recorded as data. As more is known about FANCD2 it is increasingly likely that newly identified interacting proteins may have a less visible effect on the cell, but that does not bar these new interactors from playing a profound or underappreciated role within the pathway.

FANCD2 may Interact with the Structural Maintenance of Chromosomes Proteins

The mass spectrometry data show a strong likelihood that FANCD2 is involved with the structural maintenance of chromosomes proteins in some way. SMC3 is one of the few proteins that were detected in both the endogenous and patient systems (**Table2, Table 7**). Cohesin has been shown to be enriched at sites of stalled forks and the SMC proteins SMC1A, SMC3, SMC4, SMC5, and STAG2, have previously been detected as FANCD2 interacting candidates (Lossaint 2013). Knockdown for FA proteins and SMC proteins share a genomic instability and improper segregation phenotype (Nalepa 2014, Hirano 2012). With the connection to genomic instability the cohesin and condensin complexes as well as the SMC5-SMC6 complex make excellent potential candidates for FANCD2 interactors. These proteins are also highly involved in chromatin architecture restructuring which may be a critical component to understanding how the FA-BRCA pathway becomes activated

upon damage detection. While the SMC proteins have been implicated in DNA repair the mechanism by which they act to repair DNA is currently unknown.

FANCD2 may Interact with the SWI/SNF Proteins

The SWI/SNF proteins are known as both nucleosome remodelers and transcription factors (Lu 2013). As a known tumor suppressor and the large number of complex members identified (**Table 3**), the SWI/SNF proteins make strong candidates for evaluation. The affect that the SWI/SNF proteins have on FANCD2 protein levels may be of great importance and it may provide a mechanism by which the SWI/SNF cells act as tumor suppressors. It is important to continue to evaluate the relationship between FANCD2 and the SWI/SNF proteins. There are still many metrics for characterization to look at such as chromatin localization and FANCD2 nuclear foci formation.

FANCD2 and Histones

Another notable interaction identified by this study is the interaction between FANCD2 and histones. Several histone variants were identified as the candidates however it is difficult to determine the strength of this interaction as the peptides also showed several spectral counts in the control sample, albeit at a lower frequency (**Table 3**). There are several possible reasons for this perceived interaction with histones, it may be an indirect interaction between nucleosome remodeling proteins that interact with FANCD2 or it may be an artifact of FANCD2 associating with

chromatin. However this does not preclude the possibility that FANCD2 may be directly interacting with histones.

The idea that FANCD2 may be interacting with histone is supported by the premise that FANCD2 acts as a histone chaperone (Sato 2012). This idea is further evidenced by the discovery of a possible histone binding domain within FANCD2 (unpublished data). With the difference in some of the histone peptides detected in this experiment exceeding a fourfold change in some cases it is reasonable to believe that chromatin associated FANCD2 may have a direct interaction with histones. Enrichment is shown for Histone H1, Histone H2A, Histone H2B, Histone H3, and Histone H4 (**Table 3**). It is important to realize that DNA repair occurs within the context of chromatin and while detecting interactions with histones the abundance of posttranslational modifications and histone variants remains unknown and is not likely to be decoded with such a broad screening method.

The diversity and prevalence of histones and histone remodeling proteins within the data allude to the idea that FANCD2 may be involved in binding specific chromatin marks or in causing changes in the profile of chromatin modifications. The FA-BRCA pathway may be regulated by the histone code or alternatively may be actively involved in writing the histone code. With several of the proteins in the FA core complex being understudied it is possible that the FA core complex along with FANCD2 may have a role in recognizing and modifying structures in chromatin either is conjunction with or separate from the ability for the FA-BRCA pathway to promote repair of the highly genotoxic ICL lesions.

Conclusions

This project has demonstrated a successful method to enrich for chromatin associated active FANCD2 immune complexes, and has given large clues as to which proteins compose the activated FANCD2 immune complexes. While this data shows a dramatic increase in the information known about possible FANCD2 interacting proteins, the data still requires a great deal of investigation to be performed. The methods and data described here show the massive power associated with proteomics techniques. The discovery of several strong candidate interacting proteins as well as preliminary data to characterize the interaction should contribute to understanding the activity and regulation of FANCD2 and the larger FA-BRCA pathway function in a meaningful way. Hopefully the information generated in the screen can contribute to the improvement for healthcare options of FA patients and increase the understanding of how DNA repair is mediated within the cell.

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