SAN1 – a Novel Nuclease Involved in the Repair of Interstrand Cross-links

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This work is dedicated to my parents, who have been unwavering in their support for me throughout my life and graduate school.

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LIST OF TERMS AND ABBREVIATIONS

AOA2 – ataxia with oculomotor apraxia type 2

ALS4 – juvenile amyotrophic lateral sclerosis type 4

APE1 – AP-endonuclease 1

ARTEMIS – DNA cross-link repair 1C

ATM – Ataxia Telangiectasia Mutated

ATR – Ataxia Telangiectasia and Rad3- related protein

ATRIP – ATR interacting protein

BRCA1 – Breast And Ovarian Cancer Susceptibility Protein 1

BRCA2 – Breast And Ovarian Cancer Susceptibility Protein 2

BER – Base excision repair

BLM – Bloom syndrome RecQ like helicase

BRDU - Bromodeoxyuridine

CCPG – constitutive co-activator of peroxisome proliferator-activated receptor gamma

CDK – cyclin dependent kinase

Chk1 – checkpoint kinase 1

Chk2 – checkpoint kinase 2

CPT – Camptothecin

CRISPR – Clustered regularly interspaced short palindromic repeats

CSA – Colony survival assay

CSB – Cockayne syndrome protein B

CtiP – CTBP-interacting protein

DDR – DNA damage response

DEB - Diepoxybutane

DNA2 – DNA replication helicase/nuclease 2

DNA-PK – DNA dependent protein kinase

DSB – double strand break

DUB – Deubiquitylating enzyme

EMSA – electrophoretic mobility shift assay

ERCC1 – Excision repair cross-complementing rodent repair deficiency

EXO1 – Exonuclease 1

FA – Fanconi anemia

FAK – Focal adhesion kinase

FANC – Fanconi anemia complementation group

FAN1 – FANCD2 associated nuclease 1

FEN1 – Flap endonuclease 1

GEN1 – Gen endonuclease homolog 1

GG- NER – Global genome nucleotide excision repair

gRNA – guide RNA

H2AX – H2A histone family member x

HEK – Human embryonic kidney

HJ – Holliday Junction

HR – homologous recombination

ICL – Interstrand cross-link

IF – immunofluorescence

IDL – Insertion/deletion loop

KIN – karyomegalic interstitial nephritis

LP-BER – Long patch base excision repair

MEFs – mouse embryonic fibroblasts

MMC – Mitomycin c

MMR – Mismatch repair

MMS – Methyl methane sulfate

MRN – Mre11-Rad50-Nbsa complex

MRE11 – MRE11 homolog, double strand break repair nuclease

MSH – MutS homolog

MSI – Microsatellite instability

MUS81 – MUS81 structure-specific endonuclease subunit

MUTYH – MutY DNA Glycosylase

Nbs1 – Nijmegen breakage syndrome protein 1

NER – Nucleotide excision repair

NHEJ – non-homologous end-joining

ORF – Open reading frame

PCNA – Proliferating cell nuclear antigen

PIAS1 – protein inhibitor of activated STAT 1

PTM – Post-translational modification

RAD50 – RAD50 double strand break repair protein

ROS – Reactive oxygen species

RPA – Replication protein A

SAN1 – Senataxin associated nuclease 1

SETX – Senataxin

SID – Shared interaction domain

siRNA – short interfering RNA

shRNA – short hairpin RNA

SLX1 – Structure-specific endonuclease subunit 1

SLX4 – Structure-specific endonuclease subunit SLX4

SNM1a – SNM1 homolog A

SSB – single strand break

ssDNA – single stranded DNA

SUMO – Small ubiquitin like modifier

TOPOIII α - Topoisomerase III Alpha

TC-NER – Transcription coupled nucleotide excision repair

TFIIH – Transcription factor II human

TLS – Trans lesion synthesis

USP – Ubiquitin specific peptidase 1

UV – ultraviolet

XPF – Xeroderma Pigmentosum, Complementation Group F

XPG - Xeroderma Pigmentosum, Complementation Group G

XRCC4 – X-ray repair complementing defective in chinse hamster cells 4

Y2H – Yeast two hybrid

53BP1 – P53-Binding Protein

CHAPTER I

INTRODUCTION TO THE DNA DAMAGE RESPONSE AND IDENTIFICATION OF THE NUCLEASE SAN1

Overview

Cells constantly endure an ongoing assault on the integrity of their genomes from both endogenous and exogenous sources. These insults include DNA damaging agents such as toxins, cytotoxic chemotherapeutic drugs, ionizing radiation, and UV light. Additionally cells regularly encounter compounds generated intracellularly from normal biological processes that can also pose a threat, including reactive oxygen species (ROS) and aldehydes (Jackson and Bartek et al., 2009). To maintain the integrity of the genome, organisms have evolved a complex set of pathways that possess the ability to sense and repair DNA damage, collectively referred to as the DNA damage response (DDR) (Ciccia and Elledge et al., 2010). Impairment of the function of these pathways can lead to dire consequences including genomic instability, mutations, and diseases such as cancer.

The DNA damage response consists of proteins involved in three central processes that are required for genome fidelity, regardless of the specific pathway or sub-pathway within which the proteins function. First, following the formation of a lesion, damaged DNA must be recognized by proteins known as sensors. Second, after recognition of DNA damage the appropriate repair pathway must be activated through signaling cascades, a process often carried out by kinases and various enzymes capable of post-translationally modifying other proteins (Harper and Elledge et al., 2007). These proteins are commonly referred to as transducers. Third, following activation of a pathway, the damaged DNA and lesion must be directly repaired or removed, and

replaced in a manner that limits mutation by proteins known as effectors. The effector proteins include nucleases that can recognize various structures in a specific manner and aid in the removal of damaged DNA. DNA polymerases make up another large subset of effector proteins, and are responsible for replacing the removed nucleotides or in some circumstances bypassing a lesion to allow replication to proceed (Harper and Elledge et al., 2007).

The DDR is highly regulated by cell cycle stage and checkpoints. In addition to the specific type of DNA damage that occurs, another driving factor for repair pathway choice and activation is the stage of the cell cycle in which the lesion occurs and is detected. Cell cycle checkpoints that occur throughout the progression of the cell cycle allow for the recognition of DNA damage, subsequent repair, and eventual progression. If DNA damage is too severe and cannot be repaired, checkpoints can lead to apoptosis, cell cycle arrest, or senescence (Branzei and Foiani et al., 2008).

The orchestration of cell cycle checkpoints occurs through the actions of CDKs, cyclin levels, and the activities of the phosphoinositide 3-kinase related kinases ATM, ATR, and DNA-PK (Blackford and Jackson et al., 2017). These kinases are master regulators of the DDR and have hundreds of downstream targets, notable among them the effector kinases Chk1 and Chk2. ATM is primarily activated in response to double strand breaks (DSBs) through an interaction with the MRN complex, which consists of the nuclease MRE11, Rad50, and NBS1, and plays an important role in initiating homologous recombination (HR) (Uziel et al., 2003). In contrast, ATR is mostly activated in response to replication stress and stretches of single stranded DNA (ssDNA) that have been coated with replication protein A (RPA). This interaction between ATR

and RPA is facilitated through its binding partner ATRIP, and leads to the subsequent phosphorylation of Chk1 and cell cycle arrest so that repair can occur (Cortez et al., 2001). DNA-PK, like ATM, plays an important role in the response to DSBs, particularly in the non-homologous end joining repair pathway through its interaction with the Ligase 4-XRCC4 complex (Hsu et al., 2002).

Cell cycle control of DNA repair pathway choice

One of the most well established examples of cell cycle influence on DNA repair pathway choice is the influence of cell cycle stage on HR versus NHEJ (Chapman, Taylor, and Boulton et al., 2012). These competing pathways for the repair of DSBs operate in G1 (NHEJ) or in S and G2 (HR). NHEJ is the primary pathway choice of cells in G1, largely given the requirement of a sister chromatid to serve as a homologous template in HR, which is not present until late S and G2. However the state of chromatin compaction also plays a role, and may interfere with the ability for homology search to occur in G1 when chromatin is more compact (Goodarzi, Jeggo, and Lobrich et al., 2010). The choice between these two repair pathways is also regulated by CDKs, as well as ATM and ATR. These kinases phosphorylate and activate several nucleases, including CtIP and EXO1, which influences the degree of end resection at DSBs (Wang et al., 2013) (Tomimatsu et al., 2014). Whereas a greater degree of end resection occurs in S phase and favors HR, end resection in inhibited in G1 where NHEJ occurs.

Other DNA repair pathways are simply restricted to the stage in which the type of lesions that they repair arise, for example the mismatch repair pathway that operates largely in S phase during DNA replication (Brown et al., 2002). Additionally some DNA

repair pathways function within multiple stages of the cell cycle, depending on the subset of the pathway that is activated. Nucleotide excision repair (NER) for example, plays a vital role in G1 detecting and removing bulky lesions in regions of transcribed genes. A subset of this pathway, transcription coupled NER (TC-NER), is primarily responsible for the removal of pyrimidine dimers caused by UV light, which can block transcription as well as DNA polymerases. Alternatively, global genome NER (GG-NER) operates throughout the cell cycle to detect and remove bulky lesions (Schärer et al., 2013).

In addition to the regulation of DNA repair pathways through phosphorylation by kinases including Cdk1, ATM, and ATR, the DDR response is intricately modified and controlled by ubiquitin and sumo post translational modifications (PTMs) (Jackson and Durrocher et al., 2013). Aside from K63 linked poly-ubiquitylation that controls protein degradation levels, the extent and type of numerous ubiquitylation events of proteins in the DDR has recently been determined to be an important regulatory mechanism. A well-characterized example of the importance of ubiquitylation in DNA repair is the activation and regulation of the Fanconi anemia (FA) pathway by mono-ubiquitylation (Garner and Smogorzewska et al., 2011). The FA pathway, which acts as the principle pathway for the repair of interstrand cross-links, is tightly controlled and activated by the ubiquitylation of FANCD2 and FANCI, which form a heterodimeric complex (Williams et al., 2011). Additionally the spatiotemporal removal of this ubiquitylation event is also an important regulatory step that is carried out by USP1, a deubiquitylating enzyme (DUB) (Nijman et al., 2005). Notably, failure to remove ubiquitin from FANCD2-FANCI, as well as depletion of USP1, affects sensitivity to cross-linking agents. The ubiquitylation of the histone variant H2AX, has also been shown to be important for recruitment of DNA

repair factors and chromatin dynamics as well under varying circumstances (Ikura et al., 2007).

Like ubiquitylation, sumoylation has also recently been identified to play several regulatory roles in the DDR (Dou et al., 2011). Sumoylation involves the transfer of small ubiquitin like modifier (SUMO) protein to a target protein using an E3 ligase in a mechanism similar to ubiquitylation. The replication clamp protein PCNA in fact, is modified by both sumoylation and ubiquitylation to facilitate different repair mechanisms (Niimi et al., 2008) (Parker et al., 2008). The ubiquitylation of PCNA has been to shown to recruit the binding and activities of several trans-lesion synthesis (TLS) polymerases that allow for damaged DNA to be bypassed so replication can occur, whereas the role of sumoylation is less well-defined (Chen et al., 2011). Interesting, some evidence suggest that the ubquitylation of PCNA can also lead to the recruitment of SNM1a, a nuclease important for interstrand cross-link repair (Yang et al., 2010).

DNA repair pathways within the DDR

Collectively the DNA damage response consists of numerous pathways and sub-pathways that possess the ability to detect and repair very specific types of lesions during various stages of the cell cycle (Reinhardt and Yaffe et al., 2013). These lesions include single strand breaks (SSBs), double strand breaks (DSBs), bulky lesions such as thymidine dimers or interstrand cross-links (ICLs), and mismatched base pairs. The central pathways for repairing DNA damage consist of homologous recombination (HR) and non-homologous end joining (NHEJ), the two main pathways for the repair of DSBs, as well base excision repair (BER), nucleotide excision repair (NER), and mismatch

repair (MMR) (Ciccia and Elledge et al., 2010). Multiple sub-pathways exist within these core DDR mechanisms, as well as pathways that regulate and manage less common or unique types of genomic insults. Additionally, some DNA lesions require the coordination and overlap of several DNA repair pathways cooperating and functioning together to achieve repair. Notably, the repair of interstrand cross-links, which will be discussed later in greater detail, requires the coordination of multiple repair pathways that are orchestrated by the Fanconi anemia pathway (Moldovan and D'Andrea et al., 2009).

Double strand break repair

Double strand breaks (DSBs) are among the most deleterious lesions a cell can encounter, with some reports that a single DSB can result in cell cycle arrest or apoptosis (Sandell et al., 1993). Due to this reason the central pathways of homologous recombination (HR) and non-homologous end joining (NHEJ), particularly HR, are critical for preventing genomic instability. Notably, the HR proteins BRCA1 and BRCA2 are tumor suppressors that have famously been identified as breast and ovarian cancer susceptibility genes (Powell and Kachnic et al., 2003). DSBs can be produced by ionizing radiation (IR), reactive oxygen species (ROS), and numerous chemotherapeutic agents such as Doxorubicin and the topoisomerase II inhibitor Etoposide (Mehta and Haber et al., 2014). Additionally, chemotherapeutic agents that induce interstrand cross-links (ICLs) such as Cisplatin and Mitomycin C (MMC) can also indirectly lead to the formation of DSBs (Frankenberger-Schwager et al., 2005) (Lee et al., 2006). Failure to repair DSBs as well improper DSB repair pathway choice can lead to severe

consequences such as chromosomal translocations, mutations, and apoptosis (Pastinik et al., 1999).

A critical process necessary for the proper repair of DSBs is the pathway choice between HR and NHEJ. HR is a much less error prone mechanism of repair, however it is much slower and requires a homologous template (Mao et al., 2008). The shunting of DSBs to one of these two central repair pathways is regulated on several levels including the stage of the cell cycle. NHEJ operates as the central pathway in G1, whereas DSBs are predominantly repaired by HR in S and G2 phases of the cell cycle when the presence of a sister chromatid can act as a homologous template that is required for repair (Chapman, Taylor, and Boulton et al., 2012). Another critical factor that determines pathway choice between HR and NHEJ is the process of end resection. For HR to take place, the generation of long stretches of 3' ssDNA overhangs is needed. In contrast, minimal processing of DSB ends is required for NHEJ to occur. Therefore the nucleases that are responsible for the processing of DSBs, including MRE11, CtIP, EXO1, DNA2, and ARTEMIS, are highly regulated and also influence pathway choice to a high degree (Shibata et al., 2014) (Biehs et al., 2017).

Repair by HR is initiated by the binding of DNA ends by the MRN complex, consisting of the nuclease MRE11, and the proteins RAD50 and Nbs1. After initial short distance end resection by the nucleases MRE11 and CtIP, HR pathway choice is cemented following extensive 5' to 3' resection, which occurs by nucleases including Exo1 and DNA2 (Makharashvili et al., 2014) (Nimokar et al., 2011). The resulting 3'ssDNA overhangs are then quickly bound by the trimeric ssDNA binding protein RPA. BRCA2 then participates in stimulating the exchange of RPA for the recombinase

RAD51, which is responsible for carrying out the homology search step of HR (Jensen et al., 2010) (Renkawitz, Lademann, and Jentz et al., 2014). Upon discovery of homologous sequence, the formation of D-loop structures occurs, followed by DNA synthesis and extension by DNA polymerases, which generates a Holliday junction (HJ) (Sebesta et al., 2013). HJs are then resolved by the Bloom syndrome protein BLM in complex with TOPOIIIα, leading to non-crossover products in a step termed dissolution (Raynard et al., 2006). Alternatively HJs can also be resolved by nucleases such as the SLX1-SLX4 complex, or the HJ resolvase GEN1, a member of the FEN1 family of structure specific nucleases, leading to crossover product formation (Wyatt et al., 2014) (Chan et al., 2015). Given the use of the sister chromatid as a homologous template, HR is a high fidelity DSB repair mechanism which minimizes the occurrence of mutations (Mao et al., 2008).

In contrast NHEJ is a much faster DSB repair process that requires minimal end resection. NHEJ is initiated by the binding and recognition of a DSB by the very abundant Ku70 and Ku80 proteins, usually in the G1 phase of the cell cycle (Mari et al., 2006). One factor that influences pathway choice between HR and NHEJ is initial recognition and capture of DSB ends. Therefore the high abundance of the Ku70/80 dimer provides an advantage to shunting DSB towards NHEJ in many circumstances. Upon end capture by Ku70/80, the serine threonine kinase DNA-PK is recruited leading to further activation of the pathway (Reynolds et al., 2015). In contrast to HR, minimal nuclease processing is required for NHEJ to occur, with the nuclease ARTEMIS playing an important role (Drouet et al., 2006). Finally direct ligation occurs by DNA ligase IV in complex with XRCC4, a process stimulated by the factor XLF (Ahnesorg et al., 2006). As NHEJ does not utilize a homologous donor template to orchestrate repair like HR,

NHEJ is a much more error prone repair process that can lead to small insertions, deletions, and mutations at the sites of DSBs.

Nucleotide Excision Repair

In addition to encountering and repairing double strand breaks, cells must also respond to bulky distorting lesions that form in the DNA duplex. These lesions can be caused by pyrimidine dimers induced by UV light, and block fundamental processes such as transcription (Rastogi et al., 2010). In order to deal with these types of commonly occurring DNA damage, cells have evolved a pathway known as nucleotide excision repair (NER). NER is divided into two distinct sub-pathways that can recognize, remove, and repair bulky and distorting lesions (Schärer et al., 2013). Global genome nucleotide excision repair (GG-NER) operates throughout the cell cycle independently of transcription or replication, and has the ability to detect and remove a wide variety of structurally distorting DNA lesions (Gillet and Schärer et al., 2006). In contrast transcription coupled NER (TC-NER) is activated upon stalling of RNA Pol II at a DNA lesion, and often removes less distorting lesions that have evaded surveillance by GG-NER but have blocked transcription from proceeding (Vermeulen and Fousteri et al., 2013). Similarly to many DNA repair pathways, GG-NER as well as TC-NER are strongly influenced by chromatin states and chromatin remodeling enzymes, as well as through ubiquitylation and phosphorylation events (Dijk et al., 2006).

Although the mechanisms of lesion recognition differs in GG-NER and TC-NER, during both the helicase activity of TFIIH complex aids in the further unwinding of the DNA near the lesion after recognition has taken place (Coin et al., 2007). The resulting

bubble-like structure at the site of the lesion then requires processing by several structure specific nucleases. These nucleases play an important role in NER. They include XPF-ERCC1, the 3' flap endonuclease that also functions in the repair of interstrand cross-links in the Fanconi anemia pathway (Gaillard et al., 2001), and XPG, a member of the FEN1 superfamily of structure specific nucleases (Zotter et al., 2006). After incisions by these nucleases have been made and the 22-30 nucleotide fragment is removed, the replication clamp protein PCNA leads to recruitment of DNA polymerases to carry out gap filling followed by DNA ligation (Ogi et al., 2010) (Moser et al., 2007).

Base Excision Repair

Another central DNA repair pathway that is essential for maintaining genome stability is base excision repair (BER). Base excision repair recognizes less distorting DNA lesions and removes bases through the use DNA glycosylases. It plays a central role in the response to oxidative DNA damage, alkylating agents, and spontaneous deamination of bases (Krokan and Bjoras et al., 2013). In general base excision repair occurs following recognition of a damaged or inappropriately modified base. The recruitment of specific glycosylases for differing bases and types of damage results in the generation of an abasic site. The presence of an abasic site then leads to the recruitment of several DNA polymerases that can act in a gap filling process, which is followed by ligation to complete repair. BER can be broken into two sub categories of short-patch and long-path BER (Robertson, Klungland, Rognes, and Leiros et al., 2009). Short-patch is the more prevalent pathway and utilizes AP-endonuclease 1 (APE1), as well as DNA polymerase β (Sukhanova et al., 2005). While short patch BER acts on single nucleotides

long patch BER operates primarily after replication has taken place on patches of 2-10 nucleotides in length.

Oxidative DNA damage is one of the most prevalent forms of DNA damage that occurs in cells, often as a result of the production of reactive oxygen species (ROS) from cellular respiration and other metabolic processes. However, it can also result from exposure to environmental toxins or as a byproduct of ionizing radiation (IR). One of the most problematic and commonly occurring DNA lesions that result from exposure to ROS is the modification of guanine residues due to its low redox potential. This leads to the formation of 8-oxoguanine, known also as 8-oxoG (Dianov et al., 1998). This modified base can lead to mispairing as it mimics a thymidine residue, and eventually leads to G-to-T transversion mutations after DNA replication. As the primary pathway that is responsible for recognizing and repairing these lesions, inactivation of several BER genes is linked to diseases including cancer (David, O'Shea, and Kundu et al., 2005). Most notably, mutations in MUTYH and other BER genes can result in predisposition to colon cancer (Schafmayer et al., 2007).

Mismatch Repair

DNA base pair mismatches are a commonly occurring potential source of mutations and genomic instability. The mismatch repair (MMR) pathway is a highly conserved DNA repair pathway that is responsible for the recognition and repair of mismatched base pairs to prevent mutations and preserve genome integrity (Jiricny et al., 2006). Inactivation of MMR pathway mechanisms leads to a hypermutation phenotype

and can cause microsatellite instability (MSI) at repeat sequences within the genome, a phenotype that is observed in numerous cancers (Liu et al., 1995).

In mammalian cells MMR is carried out by the MutS homolog (MSH) proteins that act as dimers, including the MSH2 and MSH6 heterodimer known as MutSα. This complex, as well as other MutS complexes, form clamp-like structures that can slide along DNA to detect base pair mismatches and small insertion or deletion loops (IDLs) that form (Gorman et al., 2007). The detection of these errors following DNA replication leads to the recruitment and activity of exonucleases that degrade the region containing the errors. The central nuclease involved in the 5' to 3' degradation of this process is EXO1, a FEN1 family member (Genschel et al., 2002).

Interstrand cross-link repair

One particularly deleterious type of DNA damage is an interstrand cross-link (ICL). **Inter**strand crosslinks differ significantly from **intra**strand cross-links, the latter creating a covalent bond between two bases within the same strand of DNA. In contrast interstrand cross-links covalently link bases on opposing strands of DNA, thereby blocking fundamental biological processes such as transcription and replication by preventing DNA strand separation (Deans and West et al., 2011). In addition to the inhibition of transcription and replication, failure to properly respond and remove ICLs by cells can result in severe consequences such as the formation of DSBs and chromosomal translocations. Unrepaired ICLs can also produce unique forms of ICL associated DNA damage, including radial chromosomes and high levels of chromosomal aberrations. These radial chromosome structures are unique to ICL induced DNA

damage, and form when unrepaired ICLs cause persistent replication fork stalling and collapse. The collapse of these forks result in the generation of one-sided double stranded breaks, which undergo aberrant NHEJ processes where one-sided DSBs and are joined to DSBs present on other chromosomes (Deans and West et al., 2011). In fact the toxicity of some ICL inducing compounds has been shown to be dependent upon this aberrant NHEJ process (Bunting and Nussenzweig et al., 2010).

The formation and appearance of radial chromosomes in response to cross-linking agents is also known as a "Fanconi-like phenotype", a name attributable to a rare genetic disorder known as Fanconi anemia, where patients exhibit developmental abnormalities, bone marrow failure, and a predisposition to numerous cancers (Nalepa and Clapp et al., 2018). Fanconi anemia is caused by mutations in DNA repair genes within the Fanconi anemia (FA) pathway, a replication dependent repair pathway required for the repair of ICLs. FA patient cells display hypersensitivity to a wide variety of cross-linking agents, including cisplatin and mitomycin c (MMC). In fact a diagnostic feature of FA patients is the elevated presence of radial chromosomes and aberrations in metaphase spreads of white blood cells, following exposure to MMC or Diepoxybutane (DEB) (Esmer et al., 2004). The FA pathway consists of 20 genes that have been identified to date, as well as several other associated genes that function in some capacity with the FA pathway to repair ICLs, but do not cause FA or have yet to be identified as being mutated in an FA patient (Ciccaldi, Sarangi, and D'Andrea et al., 2016). Indeed a small subset of FA patients still exist that lack a known causative mutation within the FA pathway, an issue complicated by the high degrees of genomic instability and mutational burdens that many FA patients' cells exhibit.

The Fanconi anemia pathway is the canonical repair mechanism for the removal of ICLs, and is responsible for orchestrating several distinct repair pathways to achieve this including translesion synthesis, homologous recombination, and nucleotide excision repair (Moldovan and D'Andrea et al., 2009). The FA pathway is activated when two replication forks converge on an ICL. Lesion detection involves the helicase FANCM and FAAP24, which causes activation of the core complex through inducing ATR signaling (Collis et al., 2007). As replication forks converge and the ICL is detected, this signaling then leads to recruitment of the FA core complex, which consists of 7 subunits from the genes FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCM and FANCL. The core complex functions largely as an E3 ubiquitin ligase with the subunit FANCL responsible for the ubiquitin ligase activity (Meetei et al., 2003). The core complex is responsible for the mono-ubiquitylation of both FANCD2 and FANCI, which together form the FANCD2-FANCI heterodimer (Williams et al., 2011). This step is crucial for activation of the pathway, and leads to the recruitment of SLX4 (FANCP) (Yamamoto et al., 2011). SLX4 acts largely as a scaffolding protein, and recruits several nucleases including SLX1, XPF-ERCC1, and Mus81 that contribute under differing circumstance to the unhooking and nucleolytic steps of the ICL repair process (Kim et al., 2013). XPF (FANCQ) acts as the catalytic component of the XPF-ERCC1 heterodimer, and is the most well established nuclease that participates in the unhooking step of ICL repair within the FA pathway (Bhagwat et al., 2009).

After incisions have been made surrounding the ICL and unhooking has occurred, trans-lesion polymerases including Polζ are recruited to complete DNA synthesis past the lesion (Roy and Schärer et al., 2016). The cross-linked nucleotide on

the opposite strand is then removed by nucleotide excision repair, and the resulting gap is filled and ligated (Wood et al., 2010). Following this step, the remaining DSB break that was generated after ICL unhooking is repaired by homologous recombination utilizing the homologous template of the sister chromatid, in a process in which BRCA1 plays a prominent role (Michl, Zimmer, and Tarsounas et al., 2016).

Replication independent ICL repair

Although the Fanconi anemia is the primary pathway for replication dependent repair of ICLs, recently other mechanisms have also been identified. Notably, an incision independent mechanism involving the glycosylase Neil3 has been characterized for the removal of Psoralen cross-links in a replication dependent manner (Semlow et al., 2016).

In addition to the replication dependent ICL repair pathways including FA, interstrand cross-links are also repaired by replication independent (RIR) mechanisms. However, these pathways are much less well-studied and characterized. These mechanisms are thought to act largely in G1 and outside of S phase where ICL repair is dominated by the FA pathway (Williams, Gottesman, and Gautier et al., 2013). Non-replicating and post-mitotic cells such as neurons rely on these pathways for a proper response to cross-linking agents. The replication independent repair of ICLs has been shown to in many circumstances take place as a transcription dependent repair process, which utilizes factors involved in nucleotide excision repair (Enoiu et al., 2012).

The FEN1 family of structure specific nucleases

The FEN1 family of structure specific nucleases plays critical roles in DNA repair, recombination, and replication (Lieber et al., 1997). Flap endonuclease 1 (FEN1), for which the family is named, is required for DNA replication due to its function in Okazaki fragment processing, as it recognizes and cleaves 5' flap structures via its endonuclease activity (Maga et al., 2001). However, it also plays a critical role in long patch base excision repair (LP-BER), and thus in the response to oxidative DNA damage (Asagoshi et al., 2010). Members of the FEN1 family all contain key conserved aspartic residues, which are utilized to carry out metal ion dependent catalysis of the phosphodiester backbone of DNA. Despite this, the nucleases in the family recognize very diverse substrates and display very different, and in some cases multiple, biochemical activities. EXO1, another member that functions in mismatch repair as well as end resection during HR, displays strong nuclease activity toward DNA bubble substrates (Bolderson et al., 2010) (Tran et al., 2007). In contrast GEN1 recognizes four way junctions to act on Holliday Junctions at a later stage in the HR process (Rass et al., 2010). Lastly, XPG plays a critical role in nucleotide excision repair (NER) through its activity on bubbles and 3' flaps (Zotter et al., 2006). Collectively the nucleases of the FEN1 family recognize and act on diverse DNA substrates in DNA replication and repair, allowing them to participate in the response to many different types of DNA damage that cells incur.

Identification of fam120b as 5' ssDNA exonuclease

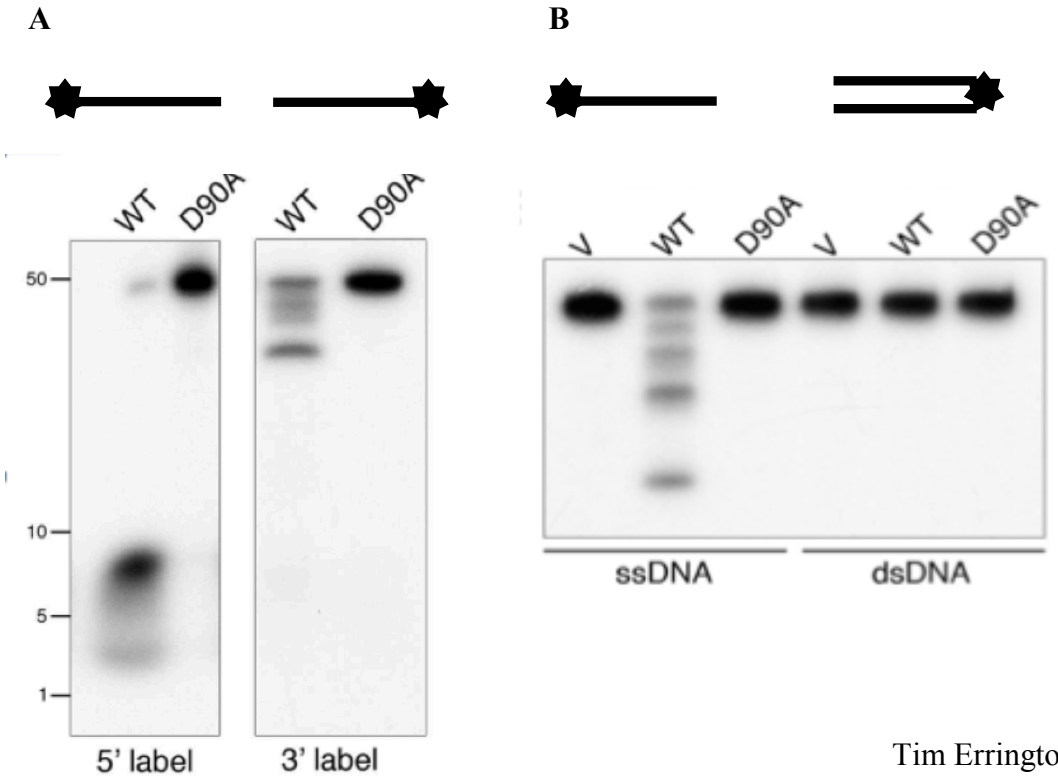
Through a proteomic screen examining potential interacting partners of septins, our lab identified a largely uncharacterized protein encoded by the gene fam120b. Septins are cytoskeletal proteins, that play roles in diverse cellular processes including acting as scaffolds, fusion barriers, and in cytokinesis (Mostowy and Cossart et al., 2012). Additionally, an unexpected link between septins and DNA repair has been previously identified (Kremer et al., 2007). However, scientific inquiries and studies described in this thesis focus on the role of SAN1 as a nuclease that functions in DNA repair, and not on the potential link to septins. Fam120b was of interest to us largely because closer examination revealed an N-terminal domain that displayed strong homology to the FEN1 superfamily of structure specific nucleases. However, to the better part of our knowledge no investigations into its potential function as a nuclease had ever previously been conducted, in fact only one paper regarding the function of fam120b had been published to date. This previous study characterized fam120b as a constitutive co-activator of peroxisome proliferator-activated receptor gamma, finding that it functions in adipogenesis and suggesting that the protein be named CCPG (Li et al., 2007). In addition to fam120b, the fam120 gene family has two additional members, fam120a and fam120c. Although there is strong sequence similarity and conservation between the family members, preliminary evidence in the literature suggest strikingly different functions for fam120a and fam120c than what we have discovered for fam120b. Fam120a has been shown to act possibly as a scaffolding protein to activate FAK and PI3K signaling (Bartolome, et al., 2015), as well to potentially play a role in RNA

binding in response to oxidative stress (Tanaka et al., 2009). Fam120c in contrast has been studied much less and may have some function as a transmembrane protein (Holden et al., 2003).

Unlike fam120b, fam120a and fam120c lack some of the conserved catalytic residues within the putative FEN1-like nuclease domain. In contrast fam120b contains all of the conserved aspartate residues present in the nuclease domains of FEN1 family members. Preliminary data from our lab suggests that fam120a and fam120c in fact do not possess nuclease activity *in vitro.* However it cannot definitely be ruled out as only ssDNA substrates were tested (data not shown).

To explore the potential function of the fam120b gene as a nuclease, we first cloned the human fam120b open reading frame (ORF) with a C-terminal FLAG tagged construct, as well as a mutant version where a conserved aspartate residue in the putative FEN1 family like domain had been mutated to an alanine (D90A). In these initial studies we referred to the fam120b gene product as SAN1, for "septin associated-nuclease 1". However later work investigating the interaction of SAN1 with the helicase senataxin, which is discussed in depth in chapter 4, has led us to propose the name of SAN1 for "senataxin-associated nuclease 1". In preliminary work carried out by Tim Errington in the lab, SAN1WT-FLAG and SAN1D90A-FLAG were purified after over-expression in HEK 293T cells and incubated with P^{32} labeled oligonucleotide substrates. Potential products were then visualized by autoradiography (Figure 1a-b). Strikingly, we found that SAN1WT displayed clear nuclease activity towards the 5' end of ssDNA substrates, where it cleaved in ~2-7 nucleotide fragments. However, SAN1 did not display activity towards dsDNA substrates, as shown in Figure 1b. Importantly, mutation of a conserved

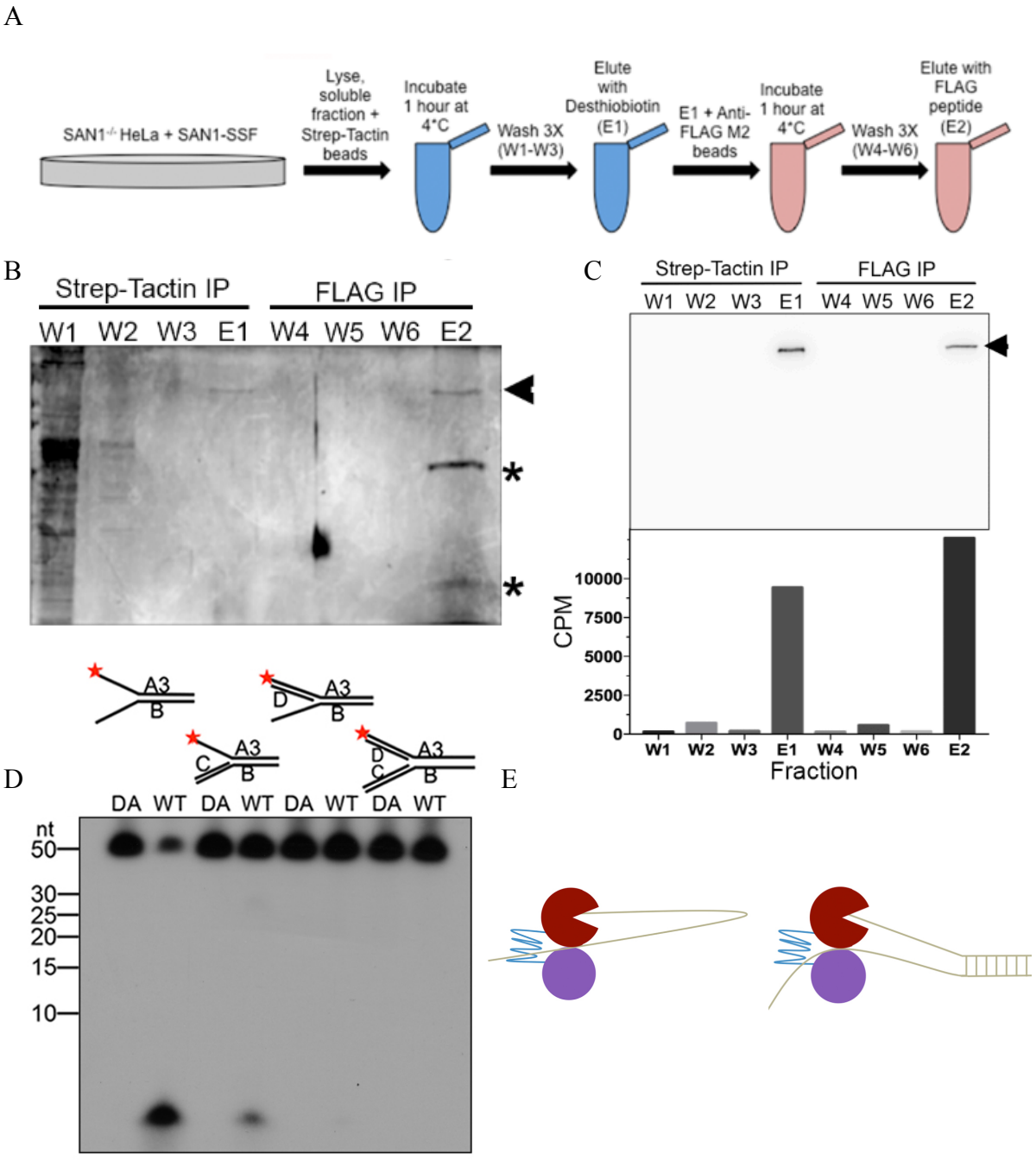
aspartate residue in SAN1 (D90A), completely blocked nuclease activity, providing evidence that the nuclease activity observed was intrinsic to SAN1, and not an artifact or contaminating nuclease. As a control we have also shown that the D90A mutant interacts and binds with DNA normally in an electrophoretic mobility shift assay (EMSA), demonstrating that the mutant is correctly folded (data not shown). From these data we were able to conclude that the gene fam120b, previously characterized as a co-activator to promote adipogenesis, in fact possesses 5' ssDNA exonuclease activity.



Tim Errington

Figure 1. SAN1 is a 5' ssDNA exonuclease.
(a) Synthetic 50-mer oligos were 5' or 3' labeled with ^{32}P and incubated with SAN1WT or SAN1D90A for 120 min. Products were separated by PAGE and ^{32}P-fragments were detected by autoradiography. (b) ssDNA or dsDNA were 5' or 3' ^{32}P labeled and incubated with WT or D90A SAN1. Products were analyzed as in (a).

However to more definitively conclude that this nuclease activity was intrinsic to SAN1, another student in the lab Heather McCartney, developed a two-step purification strategy (Figure 2a). HeLa cells over-expressing a SAN1-Strep$_2$-Flag (SAN1-ssf) construct were used to purify WT SAN1 first using streptactin beads, followed by washes and elution with desthiobiotin. This elution was then purified using anti-FLAG agarose beads before elution with flag peptide. Using this approach we showed that SAN1 was only found in the elution fractions, as shown by silver stain and western blot (Figure 2b-c). Importantly, using a spin column assay we demonstrated that nuclease activity on a 5' ssDNA substrate was only observed in those elution fractions containing SAN1, and not in any wash fractions (Figure 2c). In addition to 5' nuclease activity towards 5' ssDNA, SAN1 also showed strong activity towards 5' splayed structures, and mild activity towards 5' replication fork structures (Figure 2d).



Heather McCartney

Figure 2. Validation of intrinsic SAN1 nuclease activity.
(a) Schematic of double-affinity Strep-FLAG tag purification for human SAN1 WT and SAN1 DA. (b) Silver stained fractions from the purification where "W" denotes Wash steps and "E" denotes Elution steps for the Strep and FLAG IPs. Arrow shows human SAN1 WT (expected size 150 kD) and asterisks show FLAG antibody heavy and light chains. (c) Top panel shows immunoblot of fractions from two-step purification of SAN1

where arrow shows SAN1 (expected size 150 kD), detected using mouse M2 anti-FLAG-antibody. Bottom panel shows corresponding filter spin nuclease assay. (d) FLAG-tagged SAN1 WT or D90A was incubated with 5' ^{32}P labeled splayed duplex, 3' flap, or 5' flap structures for 2 hrs at 37°C. (e) Schematic of a model for SAN1 nuclease activity. SAN1 acts on ssDNA substrates by recognizing the free 5' end of DNA and cleaving ~3 or ~7 nts from the 5' end, in a non-processive manner.

Numerous investigations have been carried out regarding the biochemical structure and function of SAN1 as a nuclease, the bulk of which work was performed by graduate student Heather McCartney in the lab. These experiments are not discussed in depth in this thesis, which focuses instead on questions concerning the type of DNA repair in which SAN1 functions, and the proteins, pathways, and mechanisms through which it does so. The biochemical experiments have informed us with valuable information that hint at SAN1 function in DNA repair, including findings that SAN1 requires a free 5' end, displays similar kinetics to other FEN1 family members, and that the N and C -terminal domains interact (data not shown). The schematic shown in Figure 2e, illustrates some of the conclusions we have reached regarding the biochemical activity of SAN1 as a nuclease, on preferred substrates including 5' ssDNA and splayed arm structures.

CHAPTER II

SAN1 IS INVOLVED IN THE REPAIR OF INTERSTRAND CROSS-LINKS

Abstract

The findings that SAN1 is in fact a 5' ssDNA exonuclease, led us to investigate in which type of DNA repair SAN1 might function. A particularly dangerous type of DNA damage, an interstrand cross-link (ICL), was of particular interest given preliminary data from the lab. Using knockout CRISPR/Cas9 generated SAN1-/- HeLa cells, as well as SAN1-/- mouse embryonic fibroblasts (MEFs), we confirmed that the loss of SAN1 results in specific sensitivity of cells to interstrand cross-linking agents. Importantly, we observed that nuclease activity of SAN1 was required for SAN1 function in response to ICLs, implicating this protein directly in the repair process. A hallmark of Fanconi anemia and cells defective in ICL repair, radial chromosome structures, were found to be elevated in SAN1-/- cells following treatment with MMC. These data provide direct evidence of unrepaired ICLs that persist after the loss of SAN1. In agreement with these findings we also observed that SAN1-/- cells show elevated levels of DNA damage and double strand breaks (DSBs) as shown by the DNA damage response markers γH2AX and 53BP1. Collectively, the studies in this chapter provide strong evidence for the involvement of SAN1 in the repair of ICLs.

Introduction

Members of the FEN1 family play very diverse roles in DNA replication and repair, as they have the ability recognize and act as nucleases on distinct DNA structures. These structures range from flaps that are cleaved by FEN1 during Okazaki fragment

processing, to Holliday Junctions that are resolved by GEN1, as well as 5' to 3' dsDNA resection carried out by EXO1 during homologous recombination (Lieber et al., 1997). This diversity led us to investigate the involvement of SAN1 in various types of DNA repair. The biochemical activity of SAN1, particularly it's 5' ssDNA nuclease activity, as well as its activity towards 5' splayed arm substrates, and additional data showing that SAN1 displayed no activity on 3' ssDNA or flaps, substrates with nicks or gaps, Holliday junctions, or double-stranded DNA, were all informative about potential types of DNA lesions on which SAN1 might act (data not shown).

Homologous recombination is a process that requires nucleases for two key but distinct steps. End resection, which occurs in a 5' to 3' direction to create long patches of 3' ssDNA overhangs, and Holliday Junction resolution if these structures are not resolved by the HJ dissolution pathway that is orchestrated by the BLM-TOPOIIIα complex (Symington et al., 2014) (Raynard et al., 2006). However, since SAN1 lacked activity towards HJs and showed no activity on dsDNA like EXO1, it seemed unlikely that SAN1 might be required for HR. If SAN1 were to be involved in end resection, this would require the tight coupling of its activity to a helicase that would provide a ssDNA substrate. The lack of activity towards nicked and gapped substrates, intermediates that are found in base excision and nucleotide excision repair, also helped narrow possible repair pathways in which SAN1 may be involved (Liu et al., 2007).

The activity of SAN1 on 5' ssDNA and 5' splayed arm structures in fact suggested a potential involvement in the response to DNA repair pathways that respond to replication stress. As the helicase and polymerase activities of replication forks become uncoupled (Byun et al., 2005), this can result in the formation of 5' and 3'

ssDNA stretches as well as splayed arm like substrates. In summary these biochemical data led us to screen SAN1 deficient cells against a wide variety of DNA damaging agents, and particularly those that induce replication stress. Interstrand cross-links were a particularly intriguing candidate for the type of lesion that might require SAN1, as they exert their toxicity mainly through the blockage of replication forks, which the stalling and collapse of can lead to double strand breaks.

Results

After determining definitively that SAN1 acts as a 5' ssDNA nuclease, we next investigated in what potential types of DNA repair SAN1 might function. Previous data from the lab indicated that depletion of SAN1 by siRNA or shRNA in HeLa cells sensitized cells to the cross-linking agents MMC and Cisplatin (data not shown). However loss of SAN1 did not appear to sensitize cells to other types of DNA damage such as ionizing radiation which requires HR and NHEJ, or the alkylating agent methyl methane sulfate (MMS) which requires both base excision repair (BER) and HR (data not shown) (Essers et al., 1997). These experiments were performed using colony survival assays (CSAs), where cells are plated at low density and allowed to proliferate and form colonies over the course of 7-10 days. Additionally, the depletion of SAN1 by shRNA and siRNA respectively, also did not affect HeLa cell proliferation or cell cycle profile (data not shown).

SAN1 deficient cells are sensitive to cross-linking agents

To further investigate this potential phenotype, and to more definitively determine if SAN1 is involved in the response to interstrand cross-link (ICLs), including possibly the repair of these lesions, our lab utilized the CRISPR/Cas9 system to create a SAN1-/- HeLa cell line (Figure 1d). This cell line was created using two guide RNAs (gRNAs) to target a large portion of the fam120b gene that encodes the nuclease domain of SAN1. The creation and validation of the cell line via DNA sequencing and western blot was done by another graduate student in the lab Heather McCartney. Importantly the SAN1 knockout cell line displayed no proliferation defect compared to the HeLa WT parental cell line (data not shown).

As confirmation of our previous results with RNAi, the SAN1-/- HeLa cell line showed strong sensitivity to both MMC and Cisplatin but only a small response to ionizing radiation, demonstrating the specific involvement of SAN1in the response to cross-linking agents (Figure 1a-c). We also tested other DNA damaging agents including the topoisomerase I inhibitor Camptothecin (CPT) that causes single stranded breaks (SSBs), ultraviolet (UV) radiation that induces thymidine dimers and blocks transcription, and Hydroxyurea which causes replication stress via dNTP pool depletion (data not shown) (Hsiang et al., 1985) (Durbeej et al., 2002) (Collins et al., 1987). However, we found that the SAN1-/- cells were not hypersensitive to any of these types of DNA damaging agents, providing further evidence of the specific involvement of SAN1 in the DNA damage response to ICLs.

SAN1 nuclease activity is required for ICL resistance

An important hypothesis to test was if the nuclease activity of SAN1 is required in the cellular response to ICLs. To address this question we created SAN1-/- cell lines over-expressing either a WT version of SAN1, or a catalytically inactive mutant, where a highly conserved Aspartate residue within the active site of the nuclease domain was mutated to an Alanine (SAN1 D90A). Using a lentiviral system we created rescue lines over-expressing SAN1 $Strep_2$-FLAG-tagged (ssf) constructs at levels approximately 20x that of endogenous SAN1 (Figure 1d). The constructs localized similarly to both the cytoplasm and nucleus when visualized by immunofluorescence (IF), and the D90A mutant maintained the ability to bind ssDNA as demonstrated by an electrophoretic mobility shift assay (EMSA) (data not shown). Strikingly, in response to both Cisplatin and MMC, the WT version of SAN1 was able to fully restore resistance to ICLs whereas the catalytically inactive D90A mutant was unable to do so (Figure 1a-b). These data demonstrate that the nuclease activity of SAN1 is required for its role in the response to ICLs. This result suggests that SAN1 plays a more direct role in the process of ICL repair, given the necessity of its nuclease function, and likely rules out that SAN1 participates in an indirect process, such as the sensing of the lesion or activation of signaling cascades in the overall DDR response to an ICL.

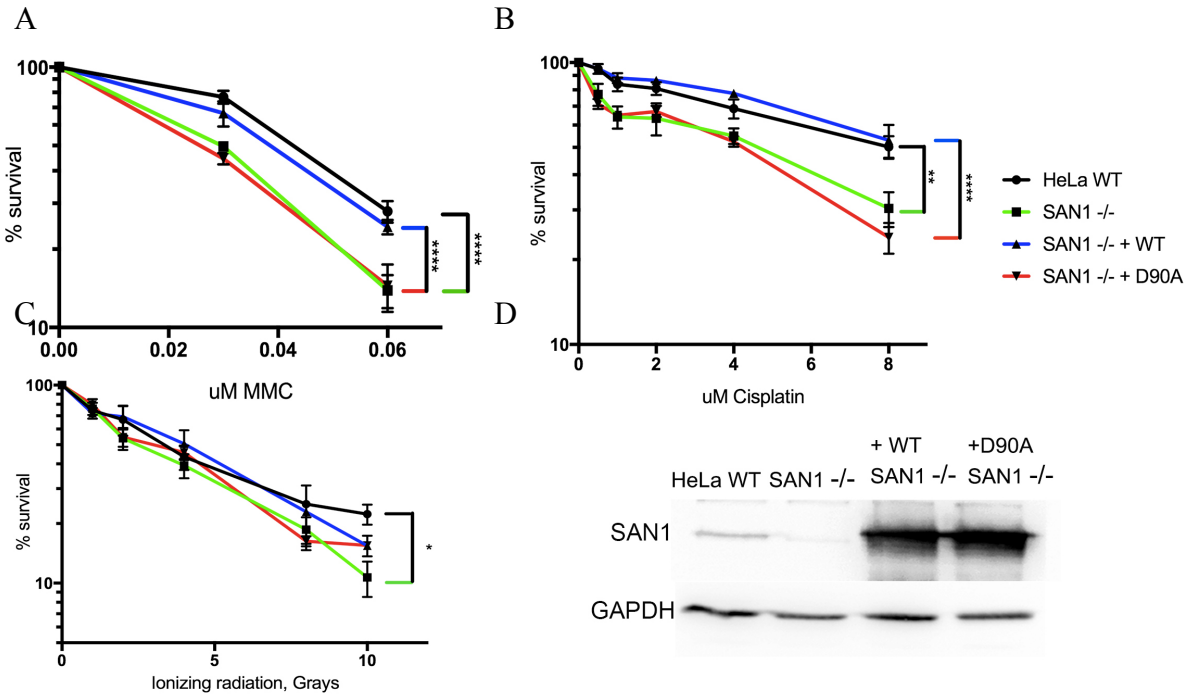


Figure 3. Loss of SAN1 leads to sensitization of cells to ICLs. (a-d) SAN1-/- cells were transduced with lentiviral constructs expressing $Strep_2$-FLAG tagged SAN1 WT or the D90A mutant to create stable rescue cell lines**.** Colony survival assays (CSAs) were then performed using HeLa WT, SAN1 -/-, and WT or D90A rescue lines with MMC, Cisplatin, or ionizing radiation ($N \geq 3$). Statistical significance determined by two-way ANOVA comparing HeLa WT and SAN1-/- or SAN1-/- +WT and SAN1-/- +D90A. Error bars denote s.e.m. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. (f) Immunoblot showing SAN1 expression in HeLa WT, SAN1 -/-, and SAN1 WT and D90A rescue lines. GAPDH was a loading control**.**

Due to the importance of this finding, we sought to validate the results using an independent methodological approach. We created a conditional KO mouse through the Texas A&M Institute for Genomic Medicine, using EUCOMM ES cells targeting the *fam120b* gene (ES cell clone HEPD0652_5_G10). A *fam120b*/+ male was crossed to a FLPer/+ female to delete the lacz/neo markers, the FLPer transgene was then removed by crossing to a +/+ mouse, and the resulting floxed allele mice were crossed to produce homozygotes. These were then crossed with a Sox2-Cre mouse to obtain a global knockout of the allele. From these SAN1-/- mice, which were viable and fertile, we

isolated primary embryonic fibroblasts. Because primary fibroblasts do not proliferate when plated as single cells, we immortalized these cells using the SV40 large T antigen, and created lines from SAN1-/- and SAN1+/- and WT +/+ littermates. To detect mouse SAN1 (mSAN1) protein by western blot, which is expressed at low endogenous levels in MEFs, we also generated and purified a polyclonal antibody using Cocalico biologicals services (Figure 2d). These cell lines were then used for CSAs. The homozygous knockout cells showed significantly increased sensitivity to ICL agents as compared to the wild type cells (Figure 2 a-b), and were not sensitive to ionizing radiation (Figure 2c). Moreover, re-introduction of human SAN1-ssf WT rescued survival but the D90A mutant did not (Figures 2a-c). In addition to validating the data from HeLa cells, showing the specific sensitivity of SAN1-/- cells to cross-linking agents, these data also suggest that the function of human and mouse SAN1 is conserved given that WT hSAN1 is able to sufficiently restore resistance to ICLs in MEFs (Figure 2a-d).

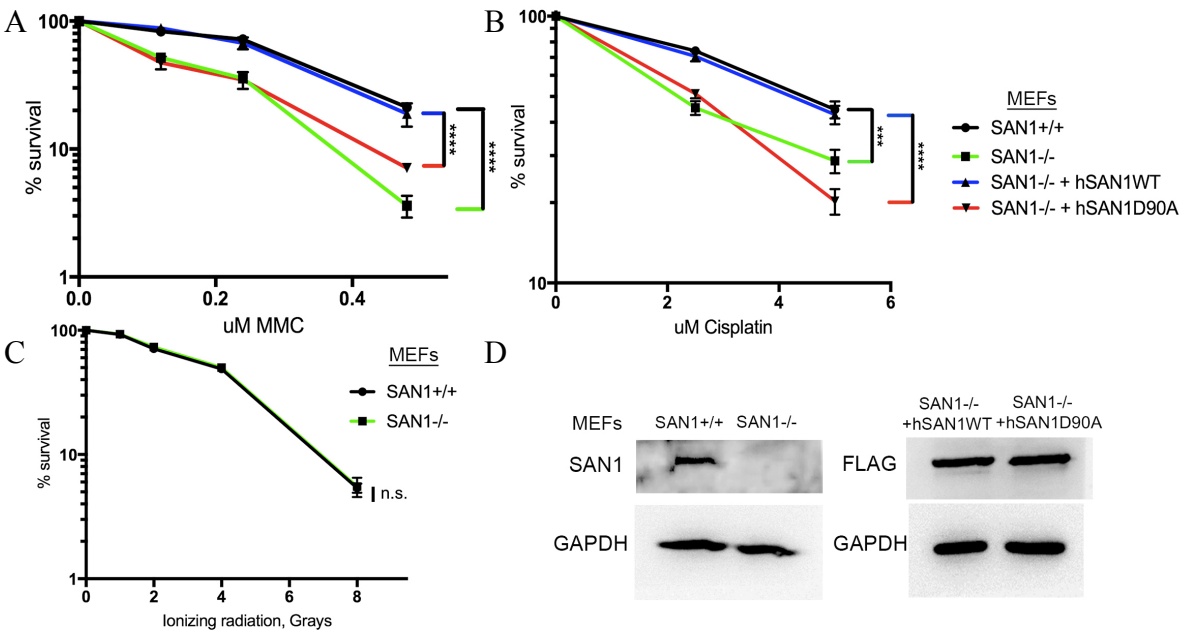


Figure 4. SAN1-/- MEFs are sensitive to cross-linking agents.

(a-d) SAN1 +/- mice were crossed to generate SAN1 +/+ and -/- mouse embryonic fibroblasts (MEFs). The MEFs were immortalized using SV40 large T antigen, and the SAN1 -/- MEFs were transduced with lentiviral constructs containing $Strep_2$-FLAG-tagged human SAN1 WT or SAN1 D90A. These cell lines were then used for CSAs with MMC, Cisplatin, or ionizing radiation (N=3). (j) Immunoblot showing mouse SAN1 expression in SAN1 +/+ and -/- MEFs (left panel) and hSAN1WT and hSAN1D90A expression in SAN1-/- cells (right panel). GAPDH was used as a loading control. Statistical significance for CSAs was determined by two-way ANOVA test comparing SAN1+/+ and SAN1-/- or SAN1-/- + hSAN1WT and SAN1-/- + hSAN1D90A.

MMC treatment induces radial chromosomes in SAN1-/- cells

We next investigated if the sensitivity of SAN1-/- cells to agents that induce ICLs was a direct result of DNA damage and a failure to repair ICLs. One type of direct evidence for a failure to respond to and repair ICLs is the appearance of radial chromosomes and chromosomal aberrations. Radial chromosomes in fact, are a diagnostic feature of Fanconi anemia, and the appearance of radials following exposure to cross-linking agents is often referred to as a Fanconi-like phenotype (Oostra et al., 2012). The formation of radial chromosomes occurs after ICLs persist and cause prolonged replication fork stalling. This can result in replication fork collapse and inappropriate processing by nucleases, generating one-sided double strand breaks that are aberrantly joined to other DSBs in the genome via NHEJ (Deans and West et al., 2011). This process can result chromosome segregation failure, as well as mitotic catastrophe and cell death. In fact evidence exists that the toxicities induced from cross-linking agents require this aberrant NHEJ process (Bunting and Nussenzweig et al., 2010).

To investigate if SAN1-/- might display these chromosomal abnormalities, we collaborated with Alan D'Andrea's lab at Harvard University. Cytogenetic analysis was performed on HeLa WT and SAN1-/- cell lines in untreated conditions as well as

following exposure to MMC. As shown in Figure 5a-b, there was no chromosomal aberrations or radial structures formed in the WT parental cell line or the SAN1-/- cells in untreated conditions. However, following exposure to 30 nM MMC, the SAN1-/- cells showed much higher levels of chromosomal breaks, aberrations, and radial structures than the HeLa WT cells, as indicated by the presence of red arrows in Figure 5c-d. The quantification of these data shows that in response to MMC there is a significant increase in both the number of aberrations per cell, as well as the percentage of cells that contain 1 or more radial structures. This Fanconi-like phenotype provides extremely strong evidence for the involvement of SAN1 in the ICL repair process. Moreover, when these data are taken into account with our discovery that SAN1 nuclease activity is required to prevent cross-link sensitivity, we can definitively conclude that SAN1 participates directly in some aspect of the ICL repair process.

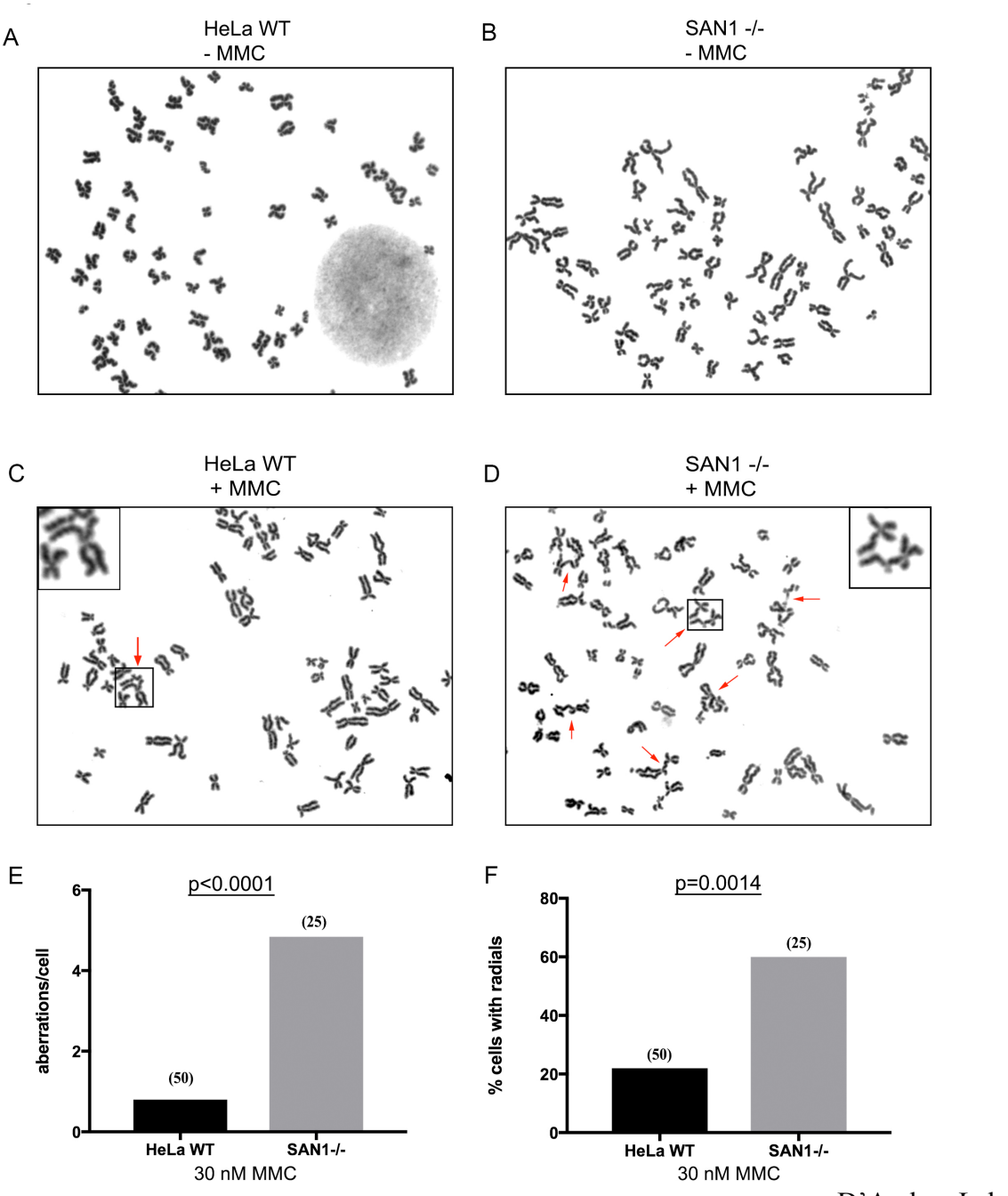


Figure 5. SAN1-/- cells display chromosomal abnormalities in response to MMC. (a, b) Micrographs of metaphase spreads from untreated HeLa WT and SAN1-/- cells. (c, d) Micrographs of metaphase spreads of HeLa WT and SAN1 -/- cells following treatment with 0.03 μM MMC, showing large increase in radials or other chromosomal aberrations in SAN1-/- cells. Red arrows indicate radial chromosomes or aberrations. (e,f) Quantification of aberrations/cell and percentage of radials/cell for HeLa WT and SAN1 -

/- cells treated with MMC. Metaphase spreads from 50 HeLa WT cells were analyzed (11 radial forms, 11 cells with radials, 40 aberrations). Metaphase spreads from 25 SAN1-/- cells were analyzed (42 radial forms, 15 cells with radials, 121 aberrations). Data were analyzed in Prism GraphPad from contingency tables using Fisher's exact-test (two-sided P value).

Loss of SAN1 causes increased DNA damage in response to ICLs

To further explore the question of if the sensitivity of SAN1-/- cells to cross-linking agents is a result of DNA damage, we examined additional DNA damage response markers. We first investigated the levels of a marker of double strand breaks and replication stress, γH2AX (Podhorecka, Skladanowski, and Bozko et al., 2010). γH2AX is key mediator of the DNA damage response and is critical for amplifying the signal of DNA damage in a cell. The histone variant H2AX is rapidly phosphorylated upon DNA damage by the master regulatory DDR kinases ATM and ATR, and the signal can spread for up to hundreds of kilobases on either side of a double stranded break (Burma et al., 2001) (Savic et al., 2009). To determine if SAN1-/- cells might display elevated γH2AX as an indicator of DNA damage, we performed a time course experiment in HeLa WT and SAN1-/- after treatment with 0.045 μM MMC (Figure 6a-b). We found that SAN1-/- cells displayed significantly elevated levels of γH2AX at both 48 and 72 hours following treatment (Figure 6a-b).

As an alternative approach, we also performed immunofluorescence experiments to measure γH2AX levels in HeLa WT and SAN1-/- cells following exposure to a higher MMC concentration for a shorter time period. Cells were treated with 1 μM MMC for 30 hours, and stained for γH2AX and DRAQ5 to mark the nucleus. As shown in Figure 6c, the intensity of γH2AX staining in SAN1-/- cells is much higher than in HeLa WT cells.

These data are quantified in Figure 6d, and show a significantly increased mean fluorescent intensity measured per nucleus from three independent experiments. Interestingly, although somewhat difficult to detect by eye in the images, the SAN1-/- cells display significantly elevated levels of γH2AX in the untreated conditions as well. This is not as striking a difference as the increase following treatment with MMC, however it is nonetheless statistically significant. One possibility is that the elevated levels of DNA damage here are the result of the formation of cross-links from endogenous sources such as aldehydes. Several types of aldehydes including acrolein, crotonaldehyde, and 4-hydroxy-2E-nonenal (HNE) have been shown to form ICLs through reactions with abasic sites (Stone et al., 2008) (Price et al., 2014). Alternatively, SAN1 might also function in other mechanisms of DNA repair independent of ICLs. The potential role of SAN1 in repair of these endogenous DNA lesions would be an important and intriguing area of future research to explore.

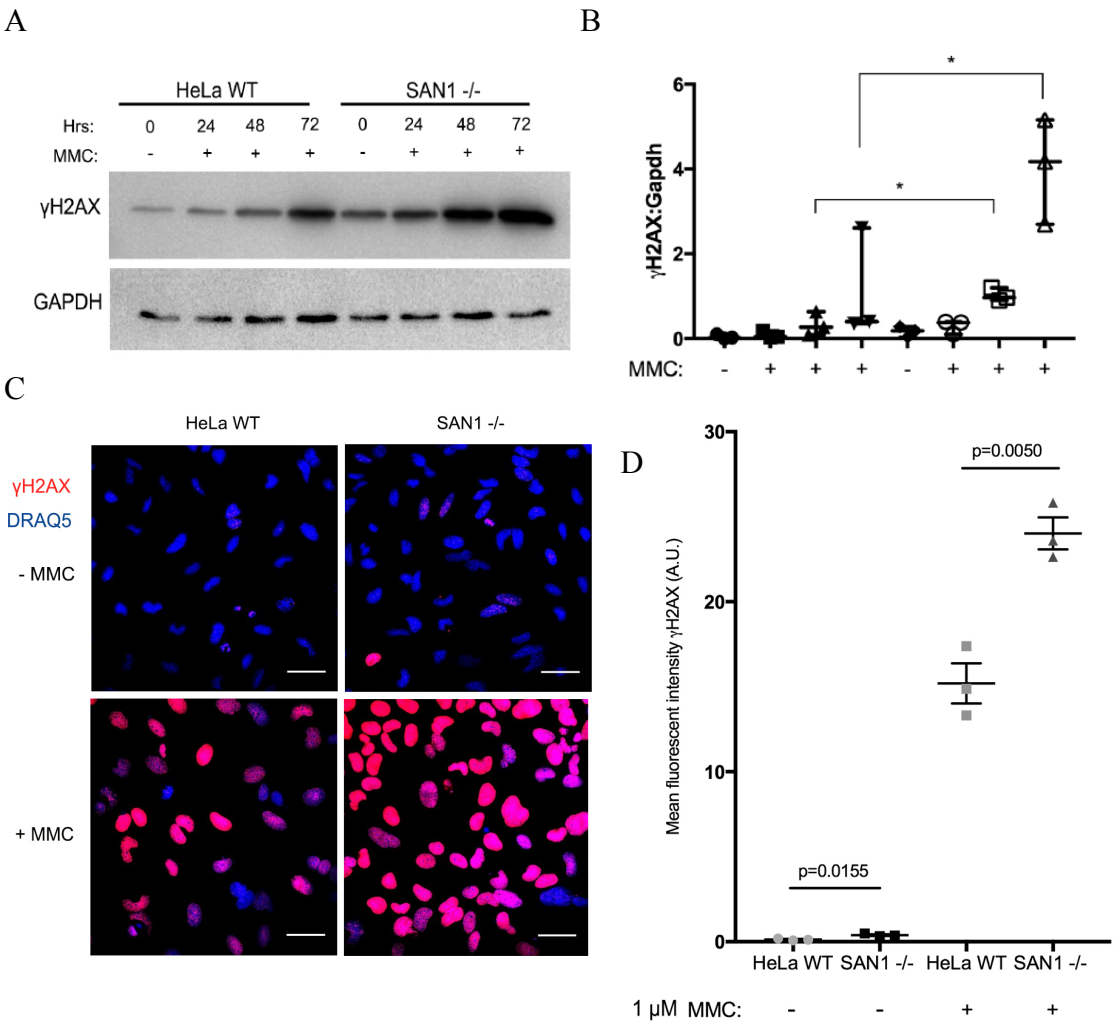


Figure 6. SAN1-/- cells have increased γH2AX in response to MMC.
(a) Immunoblots of γH2AX from HeLa WT and SAN1-/- cells treated for various times with vehicle or 0.045 μM MMC. GAPDH was a loading control. (b) Quantification of immunoblot time course (N=3). Statistical significance determined by t-test with Welch's correction. c) Immunofluorescence (IF) staining of γH2AX and DNA (Draq5) 30 hrs after treatment with vehicle or 1 μM MMC. (d) Quantification of γH2AX intensity in vehicle and MMC treated HeLa WT and SAN1-/- cells. Statistical significance determined by t-test with Welch's correction (N=3 biological replicates, at least 250 cells per sample were analyzed).

The increased γH2AX levels are in agreement with the higher levels of chromosomal aberrations and radial chromosomes that we observed. As previously

mentioned γH2AX serves as not just an indicator of DSBs, but also can be phosphorylated on Ser139 in response to replication stress (Ward et al., 2001). Both of these types of DNA damage result from ICLs, however an increase in DSBs would be theoretically required in SAN1-/- cells in order for radial chromosomes to form. Considering this possibility, we utilized an additional DNA damage response marker that is more specific to the formation of DSBs. The TP53-binding protein 1 (53BP1) is a DNA repair protein that is critical for the promotion of NHEJ, and counteracts HR through the antagonization of BRCA1 via RIF1 (Feng et al., 2013). One of the main functions of 53BP1 is the tethering of opposing ends of DNA ends at a DSB, which holds the ends in close proximity so NHEJ can occur (Difilippantonio et al., 2008). Upon DNA and a DSB induction, 53BP1 transitions from a general diffuse localization in the nucleus, to regions of discrete foci that mark the sites of DSBs (Rappold et al., 2001).

We performed immunofluorescence experiments under similar conditions as those done with γH2AX, and observed the formation of 53BP1 foci in response to MMC in HeLa WT and SAN1-/- cells. As shown in Figure 7a-b, in SAN1-/- cells we observed a striking increased in the number of 53BP1 foci present per cell, as well as the percentage of cells displaying greater than 10 foci. This is quantified in the right panel, and demonstrates that an increased number of DSBs form in response to ICLs in SAN1-/- cells, consistent with the γH2AX results and the cytogenetic analysis.

SAN1-/- cells display increased levels of double strand breaks

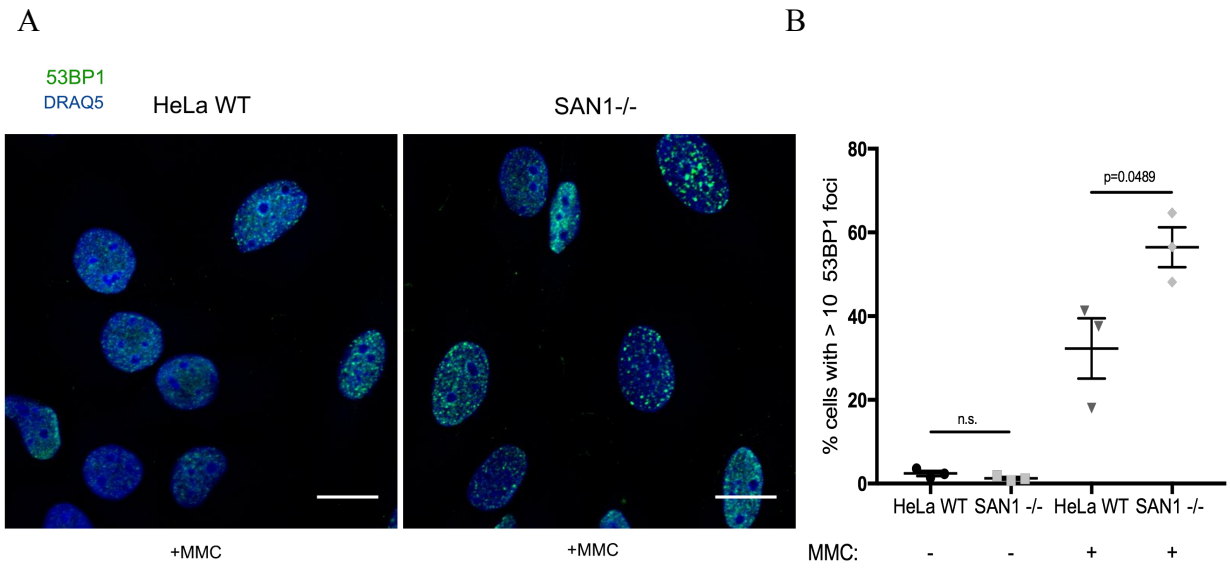


Figure 7. Treatment of SAN1-/- cells with MMC results in increased 53BP1 foci. (a) IF staining of 53BP1 and DNA (Draq5) 30 hrs post-treatment with vehicle or 1 μM MMC. (b) Quantification of percentage of cells with >10 53BP1 foci in HeLa WT and SAN1-/- cells. Statistical significance determined by unpaired t-test (N=3 biological replicates, at least 200 cells per sample were analyzed).

Discussion

To gain more insight about the potential functions of SAN1 in DNA repair, we tested a variety of DNA damaging agents that require specific repair pathways for the type of DNA lesion they create. As our lab had previously observed using RNAi approaches, SAN1-/- HeLa cells created using CRISPR/Cas9 showed strong sensitivity to ICL inducing compounds, but not other types of DNA damage. Importantly only a very mild sensitivity was observed to ionizing radiation that induces DSBs, which requires homologous recombination for repair (Essers et al., 1997). This is a critical piece of data, due to the close coordination and interconnectivity of ICL repair and HR (Michl et al., 2016). In fact the Fanconi anemia pathway that is required for ICL repair, is sometimes referred to as the Fanconi anemia/BRCA pathway, due to the requirement of the BRCA1 and BRCA2 proteins that function in HR during cross-link repair (D'Andrea and Grompe

et al., 2003). Additionally, BRCA1 (FANCS) and BRCA2 (FANCD1) have been identified as FA genes that are necessary for the repair of ICLs (Sawyer et al., 2015) (Hirsch et al., 2004). It is therefore plausible that 5' nucleases like SAN1, might function more downstream in ICL repair during the end resection step of HR. However, due to the fact that SAN1-/- cells show a lack of sensitivity to ionizing radiation in both HeLa cells and MEFs, this is highly unlikely.

A crucial finding that has furthered our understanding of SAN1 function in response to ICLs, is our demonstration that the nuclease activity of SAN1 is necessary for resistance to cross-linking agents. Moreover, that this result is conserved from human cells to MEFs provides strong evidence of the function of SAN1 in ICL repair as a nuclease.

The observation that SAN1-/- cells contain increased levels of chromosomal aberrations and radials, as well as increased γH2AX and 53BP1 foci, provides strong evidence that the sensitivity of SAN1-/- cells to cross-linking agents is a direct result of ICLs remaining unrepaired, which then results in subsequent DNA damage. The presence of radial structures in particular, is a very convincing indication of DNA damage that is specifically caused by unrepaired ICLs.

Collectively, from the studies carried out in this chapter several important conclusions can be made. First, that SAN1 is definitively involved in the response to ICLs, but not to other types of DNA damage. Second, that the nuclease activity of SAN1 is required for its function in ICL repair. Third, the sensitivity of SAN1-/- to cross-linking agents is a result of unrepaired ICLs and DNA damage, and not a consequence of an indirect effect that is caused by the loss of SAN1.

CHAPTER III

SAN1 FUNCTIONS INDEPENDENTLY OF THE FANCONI ANEMIA PATHWAY IN ICL REPAIR

Abstract

Our findings that SAN1-/- cells are specifically sensitive to ICLs, and that they display elevated levels of DNA damage and radial chromosomes, led us to further investigate the function of SAN1 in the response to cross-linking agents. In this chapter we sought to explore the potential involvement of SAN1 in canonical ICL repair mechanisms, as well as the relationship of SAN1 to other nucleases that function in interstrand cross-link repair. To determine if SAN1 functioned within the canonical ICL repair pathway, the Fanconi anemia pathway, we performed epistasis experiments where we depleted a core FA component FANCD2, and the nuclease XPF-ERCC1. We observed a strong synergistic decrease in cell survival in response to cross-linking agents in SAN1-/- cells when the FA pathway was inhibited. Additionally we found that the loss of SAN1 did not impact FA pathway activation, as evidenced by FANCD2 mono-ubiquitylation and foci formation. Collectively our results indicate that SAN1 functions independently of the canonical ICL repair pathway, the Fanconi anemia pathway in ICL repair. Our data also suggest that SAN1 functions cooperatively with the nucleases SNM1a and FAN1 in the cellular response to ICLs.

Introduction

The Fanconi anemia (FA) pathway is the canonical mechanism through which ICLs are repaired. As the FA pathway is replication dependent, it operates primarily during S and G2 phases of the cell cycle (Kim et al., 2008). The FA pathway is activated when a key protein FANCD2, is ubiquitylated by the FA core complex following detection of the ICL (Longerich et al., 2014). This activation of the pathway leads to the recruitment of SLX4, which brings several nucleases including the XPF-ERCC1 complex, that acts to unhook an ICL from the 3' side (Klein Douwel et al., 2014). However, it remains unclear if other 5' nucleases act cooperatively to participate in ICL removal after recognition and processing of a 5' flap (Zhang and Walter et al., 2014).

Two additional 5' nucleases that act partially or wholly independently of the FA pathway, also play roles in the ICL repair process. FAN1, which interacts with FANCD2, can in some circumstances act as an endonuclease and unhook ICLs from the 5' side (Pizzolato et al., 2014). SNM1a, a dsDNA exonuclease, possesses the biochemical activity to digest past an ICL, effectively unhooking the lesion in an alternative manner (Wang et al., 2011). However, the specific roles of the nucleases and the mechanisms or pathways through which they function in ICL repair remain poorly defined.

Much progress has been made in recent years defining the components and mechanisms required for ICL unhooking and removal in *in vitro* biochemical systems. The bulk of this work has been carried out in Xenopus egg extracts, and has allowed for the stepwise identification of proteins that are necessary for proper ICL repair (Räschle et al., 2008). This well-defined biochemical system has allowed for significant advances in the understanding of the spatiotemporal aspects of lesion recognition, nucleolytic

incision, trans-lesion synthesis, and the removal of ICLs within the repair process. These cell free systems have been particularly vital to understanding how the canonical pathway for ICL repair, the Fanconi anemia pathway, functions. However, much less is known about how these processes occur within mammalian cells, and clear differences persist between model systems. Notably, when the nuclease FAN1 is lost in mammalian cells a phenotype of hypersensitivity to cross-linking agents is observed (Kratz et al., 2010). In Xenopus egg extracts, however, depletion of FAN does not prevent unhooking of the ICL unlike loss of XPF-ERCC1 (Klein Douwel et al., 2014). Some of these differences may be attributable to the extra chromosomal nature of the plasmid based ICL repair system in which these studies were conducted. Clear differences also persist between mammalian systems of ICL repair, as the sensitivities of mice and human cells to cross-linking agents vary widely, as well as the fact that knockout mouse models of Fanconi anemia genes display only very mild phenotypes (Parmar, D'Andrea, and Niedernhofer et al., 2009).

Despite these caveats, knowledge of how ICLs are repaired has leapt forward during the last decade of research. As new players in ICL repair continue to be identified, more investigations are needed regarding the relationships of these proteins to the canonical ICL repair pathways. It has long been speculated that other 5' nucleases might exist but have not yet been identified, and participate in ICL repair possibly as a part of the Fanconi anemia pathway. There are several reasons for why this speculation has occurred.

Strong evidence has been presented that the FA pathway requires the dual convergence of replication forks. However, only the 3' Flap endonuclease XPF (FANCQ), has definitively been established as a nuclease that is required for unhooking

of the ICL, and as a gene that causes FA (Boglio et al., 2013). The identity of a 5' Flap endonuclease that is definitively required for unhooking has remained elusive, if it exists at all. Alternatively, one possible reason that a 5' flap endonuclease has not been identified as a FA gene that is required for the unhooking of ICLs, is that several 5' nucleases function cooperatively and act redundantly in this process. Nevertheless, several potential candidates have been identified and play at least some role as 5' nucleases in ICL repair. The best known candidate, FAN1, is also the most recently discovered 5' nuclease with a role in ICL repair. FAN1 was first identified by several groups as a 5' flap endonuclease that interacts with FANCD2, and functions in ICL repair (Smogorzewska et al., 2010) (Kratz et al., 2010) (Liu et al., 2010) (MacKay et al., 2010). While FAN1 possesses the ability to 'unhook' ICLs through its recognition of a 5' flap adjacent to an ICL, mutations in FAN1 do not in fact cause Fanconi anemia (Trujillo et al., 2012). Moreover, recent reports suggest that despite its interaction with FANCD2, it acts at least partially outside of the FA pathway, and that its interaction with FANCD2 is not required for its function in ICL repair (Thongthip et al., 2016).

Another candidate, SLX1, has long been speculated to be the 5' flap endonuclease that is responsible for ICL unhooking from the 5' side. In a similar manner as XPF-ERCC1, SLX1 also binds SLX4 and is recruited to the sites of ICLs following FANCD2-FANCI mono-ubiquitylation. The SLX1-SLX4 complex like FAN1 also possesses the biochemical ability to recognize a 5' Flap and reach past an ICL to incise and unhook the cross-link lesion (Castor et al., 2013). However, SLX1 deficient cells display only a very mild sensitivity to cross-linking agents, and SLX1 is not a gene mutated in FA (Castor et al., 2013). Lastly, the 5' nuclease SNM1a, encoded by the gene DCRE1a, has also been

implicated in the repair of ICLs. SNM1a functions independently of the FA pathway, and has implicated in both replication coupled ICL repair through an interaction with PCNA (Yang et al., 2010), as well as transcription coupled ICL repair through cooperation with the NER protein Cockayne syndrome B (CSB) (Iyama et al., 2015). SNM1a possesses a unique biochemical activity that allows it to act on dsDNA and digest past an ICL, yet SNM1a deficient cells are only somewhat mildly sensitive to cross-linking agents and its specific role in ICL repair remains very unclear (Wang et al., 2011). Collectively, the roles of the known 5' nucleases in ICL repair are complex, and many questions remain unanswered regarding the pathways that they function within and the mechanisms by which they prevent sensitivity to cross-linking agents. The discovery that another previously uncharacterized nuclease SAN1, plays a role in the response to ICLs, furthers the understanding of this important part of ICL repair. In this chapter we discuss investigations regarding the relationship of SAN1 to the canonical mechanism of ICL repair by the Fanconi anemia pathway, as well as its relationship to other nucleases involved in alternative methods of cross-link repair.

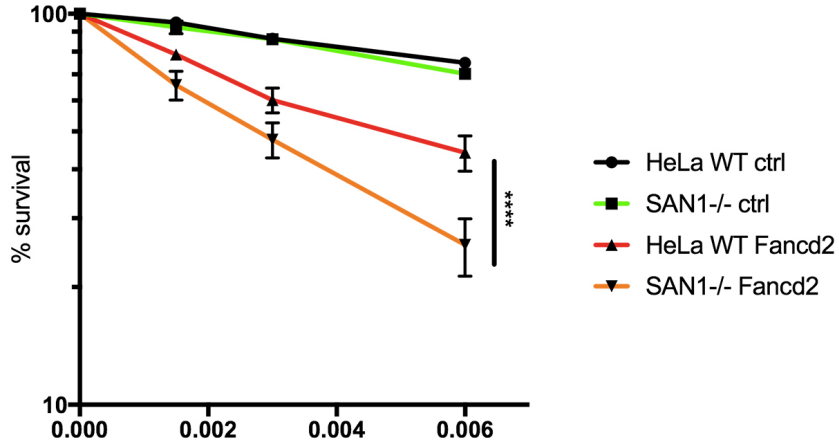
Results

SAN1 is non-epistatic to the Fanconi anemia pathway

To assess the possibility that SAN1 might function within the Fanconi anemia pathway, we performed epistasis experiments where we depleted FANCD2 in both HeLa WT and SAN1-/- cells (Figure 8). FANCD2 is a central component of the FA pathway, and its mono-ubiquitylation along with FANCI, leads to pathway activation. The formation of FANCD2 foci is thought to mark the sites of ICLs, and serve as a signal to

initiate repair of the lesions (Rothfuss and Grompe et al., 2004). Nucleases that have been shown to be associated with or function within the FA pathway such as XPF-ERCC1 (FANCQ), SLX1-SLX4, and FAN1 all function downstream of FANCD2. Therefore, if SAN1 functions within the FA pathway as a nuclease, it would almost certainly act downstream of FANCD2 in the pathway. Upon depletion of FANCD2 we observed a strong sensitivity of HeLa WT cells, at even low concentrations, to both Cisplatin and MMC as has been previously reported. Strikingly, we observed a further decrease in cell survival in colony survival assays when FANCD2 was depleted in SAN1-/- cells. In particular in response to MMC, FANCD2 depletion in SAN1-/- conferred a synergistic decrease in cell survival at these low concentrations (Figure 8a-b).

A



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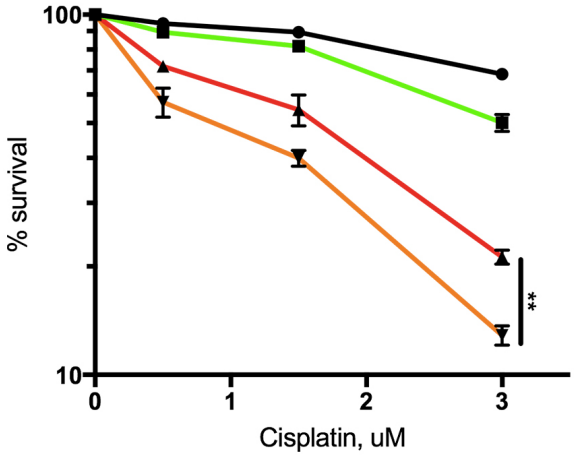

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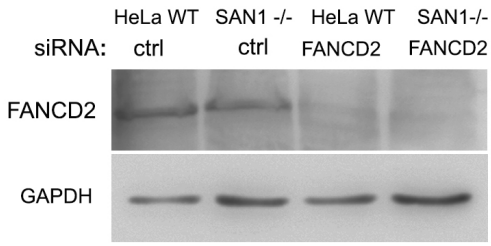


Figure 8. SAN1 functions independently of the Fanconi anemia pathway.
(a-b) CSAs of HeLa WT and SAN1 -/- cells treated with scrambled ctrl siRNA or FANCD2 siRNA, in response to Cisplatin and MMC (N=3). Statistical significance determined by two-way ANOVA. Error bars denote s.e.m. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. (c) Immunoblot showing siRNA knockdown of FANCD2 in HeLa WT and SAN1-/- cells.

SAN1 is non-epistatic to the 3' Flap endonuclease XPF

In addition to examining the relationship of SAN1 and FANCD2 in ICL repair, we also performed epistasis experiments with XPF, the catalytic component of the 3' Flap endonuclease dimer XPF-ERCC1. XPF-ERCC1 acts within the FA pathway, and is recruited to ICL sites via an interaction with the scaffolding protein SLX4 following FANCD2 mono-ubiquitylation (Klein Douwel et al., 2017). XPF is the most well established nuclease that has been shown to function within the nucleolytic steps of ICL repair, and is required for unhooking of the ICL lesion in most circumstances (Hodskinson et al., 2014). Depletion of XPF led to hypersensitivity of HeLa WT cells to low concentrations of MMC and Cisplatin, similarly to FANCD2 depletion, as has been previously shown. In agreement with the synergistic effect of FANCD2 depletion in SAN1-/- cells, loss of XPF also caused a further decrease in cell survival in response to cross-linking agents (Figure 9a-c). These data provide further evidence that SAN1 functions independently of the canonical replication-dependent mechanism for the repair of ICLs.

In these experiments we observed only a small to negligible decrease in survival between HeLa WT and SAN1-/- cells at very low concentrations of Cisplatin and MMC. This result, combined with the synergistic increase in sensitivity upon the depletion of FANCD2 and XPF in SAN1-/- cells, suggests that ICLs are predominantly processed by

the FA pathway and that SAN1 might function in a secondary pathway if the FA pathway is overwhelmed by abundant ICLs. Collectively, our data argue that that SAN1 is not epistatic to FANCD2 or XPF, and functions independently of the FA pathway

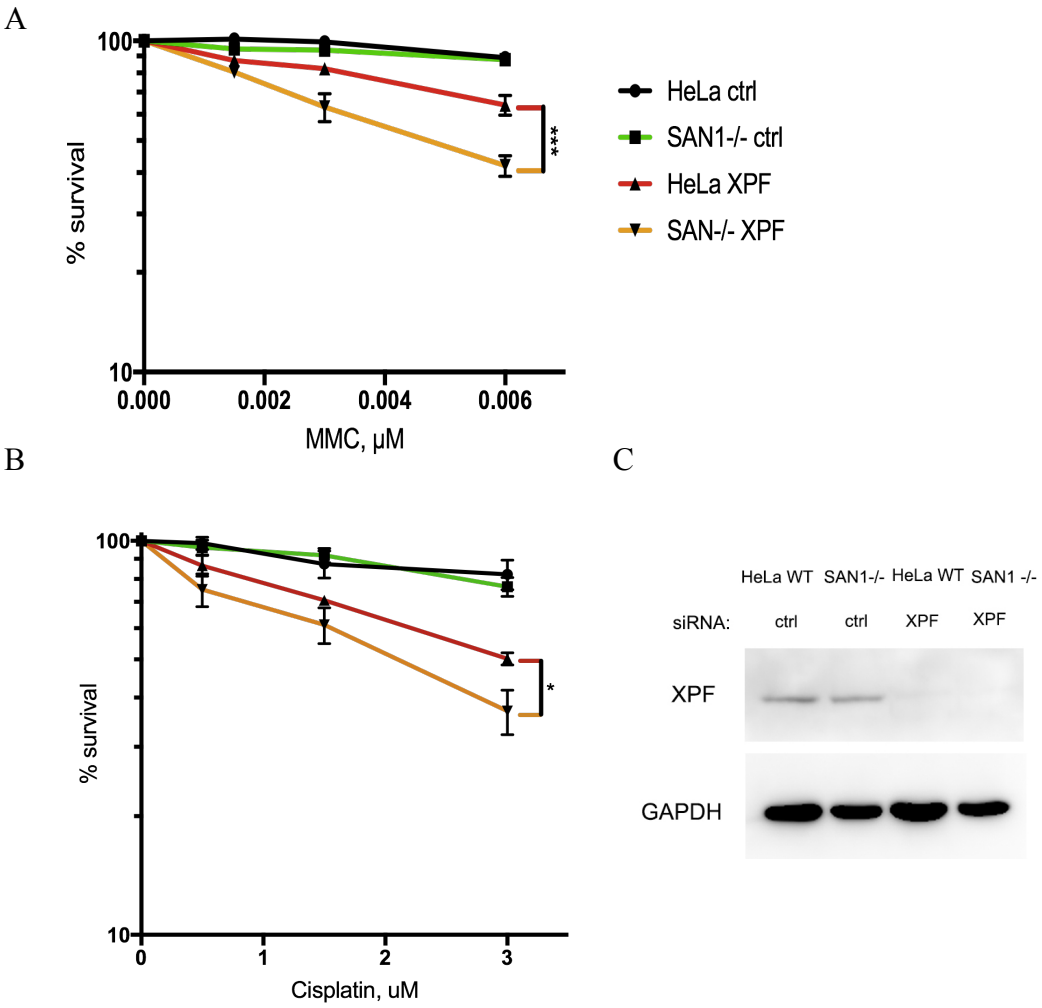


Figure 9. SAN1 functions independently of XPF in response to ICLs. (a-b) CSAs of HeLa WT and SAN1 -/- cells treated with scrambled ctrl siRNA or XPF siRNA, in response to Cisplatin and MMC (N=3) Statistical significance shown between HeLa WT and SAN1-/- +XPF siRNA conditions determined by two-way ANOVA. Error bars denote s.e.m. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. (c) Immunoblot showing siRNA knockdown of XPF in HeLa WT and SAN1-/- cells.

The Fanconi anemia pathway is intact in SAN1-/- cells

One alternative possibility that is important to consider, was that rather than SAN1 functioning independently of the Fanconi anemia pathway, the loss of SAN1 might indirectly affect FA pathway activation or fidelity, therefore causing sensitivity to ICLs. Although this is unlikely, given the requirement of SAN1 nuclease activity for resistance to cross-linking agents, it is nonetheless a necessary consideration given how critical the Fanconi anemia pathway is for ICL repair in most circumstances.

To address this question, we tested if FANCD2 was able to be mono-ubiquitylated in SAN1-/- cells following ICL formation. Importantly we observed no difference in the mono-ubiquitylation of FANCD2 following the loss of SAN1, a step that is required for FA pathway activation and for FANCD2 foci formation (Alpi et al., 2008). As shown in Figure 10b, FANCD2 presents a second higher molecular weight species (FANCD2-L) in response to MMC treatment. Since the 8 subunit FA core complex is responsible for ICL detection and ubiquitylation of FANCD2 and FANCI, these data suggest that the upstream components of the FA pathway are unaffected by the loss of SAN1.

We also directly examined the ability of FANCD2 to form foci in response to treatment with MMC in HeLa WT and SAN1-/- cells by immunofluorescence staining. FANCD2 foci serve as an important marker of the functionality of the pathway, as the foci mark ICL sites where FANCD2 localizes and subsequently recruits nucleases to participate in repair (Collis et al., 2006). Reassuringly, we observed no difference in FANCD2 foci formation in SAN1-/- cells after MMC treatment (Figure 10a, c-d). Overall, our data suggests that the Fanconi anemia pathway remains intact and functional,

ruling out the possibility that the loss of SAN1 indirectly affects the canonical ICL repair pathway.

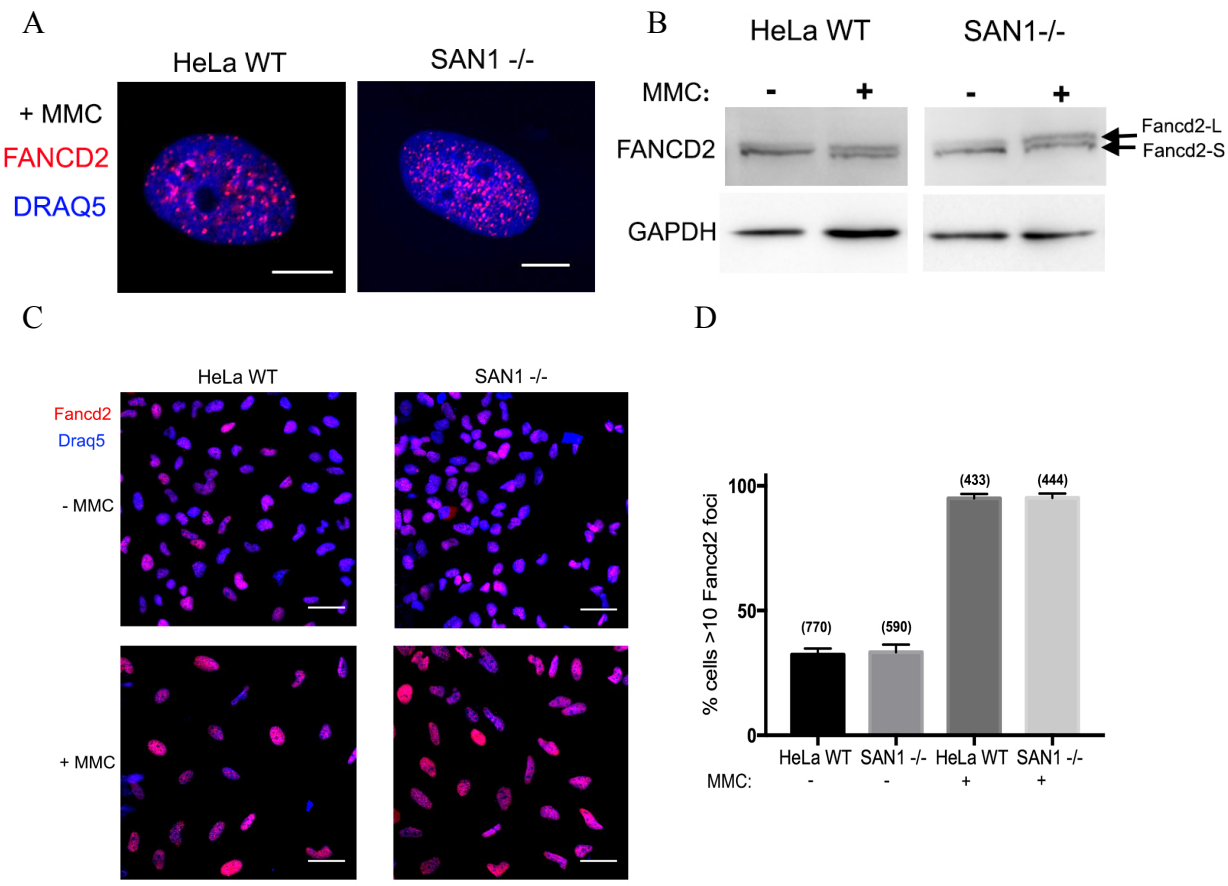


Figure 10. The Fanconi anemia pathway is intact in SAN1-/- cells. (a) IF staining of FANCD2 foci in HeLa WT cells and SAN1-/- cells treated with 0.045 μM MMC. (b) Immunoblot of FANCD2 showing mono-ubiquitylation in HeLa WT and SAN1 -/- cells treated with vehicle or 0.045 μM MMC. (d) Immunofluorescence images displaying FANCD2 foci formation in HeLa WT and SAN1-/- cells treated with vehicle or MMC. Scale bar = 50 μm. (e) Quantification of the percentage of cells with >10 FANCD2 foci in HeLa WT and SAN1-/- cells treated with vehicle or 0.045 μM MMC. (N=3 biological replicates, at least 100 cells were analyzed per sample.

SAN1 function in ICL repair is epistatic to the nucleases SNM1a and FAN1

Although the Fanconi anemia pathway is the canonical ICL repair pathway, several ICL repair mechanisms exists that operate independently from the FA pathway. These include both replication-dependent and replication-independent mechanisms of repair. Since our data strongly indicate that SAN1 functions outside of the Fanconi anemia pathway, we sought to investigate the relationship of SAN1 to other 5' nucleases that function either partially (FAN1) or wholly (SNM1a) independently of the FA pathway. The nuclease SNM1a was a particularly attractive candidate, given that SNM1a also functions outside the Fanconi anemia pathway and its specific function and mechanism through which it participates in ICL repair remains unclear (Ishiai et al., 2004). SNM1a is a member of the Metallo-β-Lactamase family of nucleases, with members including SNM1b/Apollo which is implicated in telomere protection (Touzat et al., 2010), and SNM1c/Artemis which plays a more well-established role in NHEJ (Riballo et al., 2004). Unlike SAN1, SNM1a possesses the ability to digest double stranded DNA, as well as has been shown to be able to digest past an ICL effectively unhooking the lesion from the 5' side (Wang et al., 2011). SNM1a has been implicated in transcription coupled ICL repair mechanisms (Iyama et al., 2015), as well as potentially in replication coupled ICL repair through an interaction with the DNA replication protein PCNA (Yang et al., 2010).

To test if SAN1 might function in an ICL repair mechanism with SNM1a, we performed epistasis experiments where SNM1a was depleted in HeLa WT and SAN1-/- cells. As has been previously reported, we found that loss of SNM1a in HeLa cells resulted in mild to moderate sensitivity to cross-linking agents Cisplatin and MMC, in

fact similar levels of sensitization to the SAN1-/- cell line were observed. Additionally, in striking contrast to the results we observed after depletion of FANCD2 and XPF, we observed no synergistic effect or further decrease in survival of SAN1-/- cells depleted of SNM1a in response to cross-linking agents (Figure 11a-c), suggesting therefore the possibility that SAN1 and SNM1a function cooperatively or redundantly to repair ICLs within the same pathway.

We also investigated the relationship of another 5' nuclease, FAN1, which has been shown to under some circumstances participate in the unhooking step of ICL repair, either by acting as an endonuclease on a 5' flap, or as an exonuclease that can digest past an ICL similarly to SNM1a. However, FAN1 has a complex relationship to the Fanconi anemia pathway, and its role in ICL repair is still ambiguous and disputed (Jin and Cho et al., 2017). FAN1 interacts with ubiquitylated FANCD2, however recent evidence suggests that this is not required for its function in ICL repair (Thongthip et al., 2016). Moreover, while loss of FAN1 results in strong sensitivity to cross-linking agents in human and mouse cells, patients in which the FAN1 gene has been mutated or deleted do not have Fanconi anemia (Trujillo et al., 2012). Instead, loss of FAN1 leads to a rare kidney disorder karyomegalic interstitial nephritis (KIN) (Zhou et al., 2012). We depleted FAN1 from both WT and SAN1-/- cells, and performed colony survival assays in response to MMC (Figure 11d-e). Similarly to depletion of SNM1a, the loss of FAN1 sensitized WT cells to MMC as has been previously reported (Kratz et al., 2010). Additionally, we again observed no further decrease in survival upon FAN1 depletion in SAN1-/- cells. This data suggest that SAN1 might also function cooperatively with the nuclease FAN1 in ICL repair, in a mechanism independent of the FA pathway.

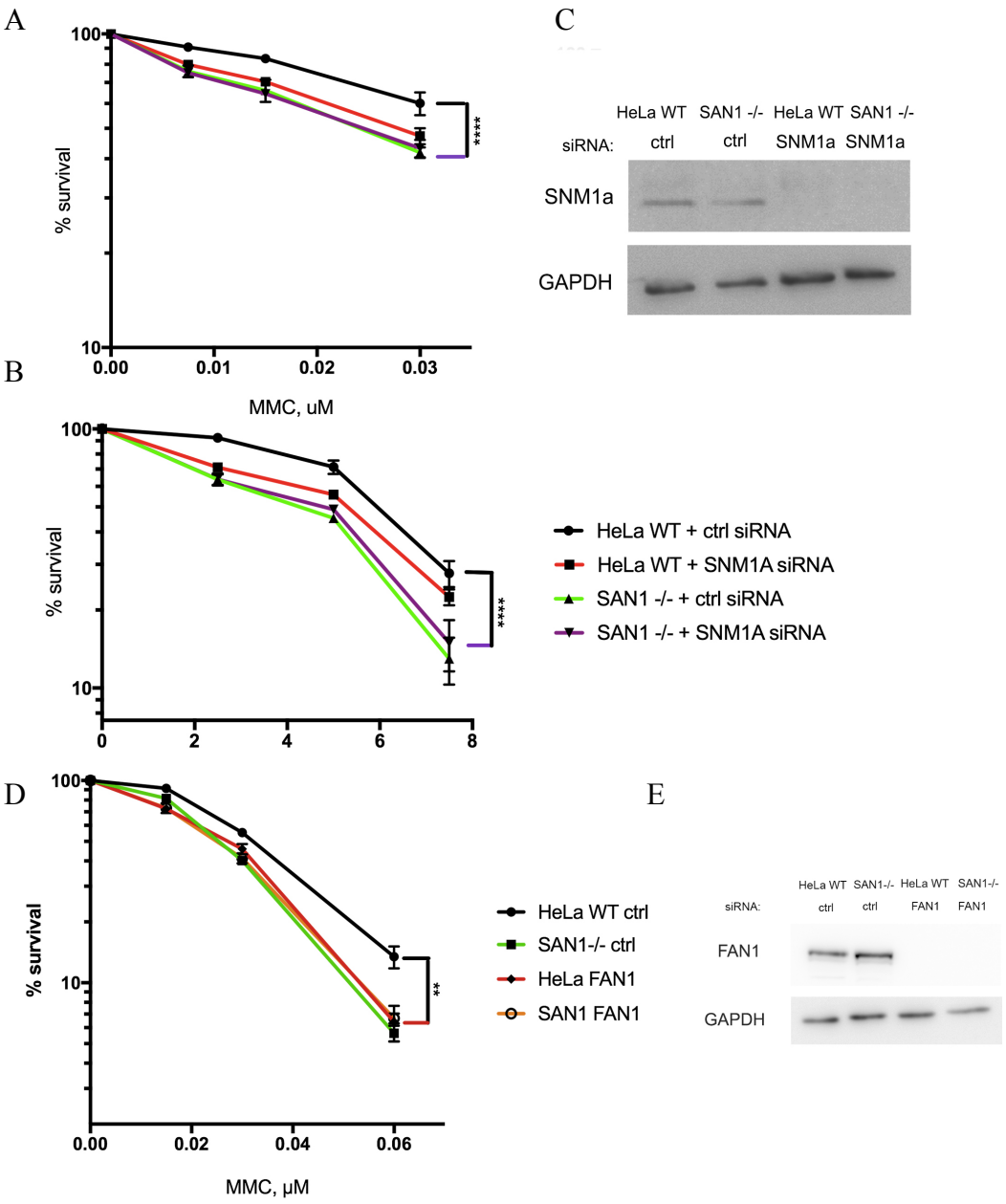


Figure 11. SAN1 is epistatic to the 5' nucleases SNM1a and FAN1.
(a-b)CSAs of HeLa WT and SAN1-/- cells treated with ctrl or SNM1A siRNA and exposed to Cisplatin or MMC. Statistical significance was determined by two-way ANOVA test. Statistical significance determined by two-way ANOVA. Error bars denote s.e.m. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. (c) Immunoblot of SNM1A in HeLa WT and SAN1-/- cells treated with ctrl or SNM1A siRNA. (d) CSA of HeLa WT and SAN1-/- cells treated with scrambled ctrl siRNA or FAN1 siRNA, in response to MMC (N=3) Statistical significance shown between HeLa WT +ctrl siRNA

and HeLa WT +FAN1 siRNA conditions determined by two-way ANOVA. (e) Immunoblot showing siRNA knockdown of FAN1 in HeLa WT and SAN1-/- cells.

Discussion

That SAN1 is non-epistatic with the Fanconi anemia pathway is not terribly surprising. The sensitivity of SAN1-/- cells to MMC and Cisplatin is more mild than that which is observed for many components of the Fanconi anemia pathway, including FANCD2 and XPF. Moreover, SAN1 has never previously been identified in any of the large scale genetic or proteomic screens that have led to the identification of ICL repair proteins and other proteins that are associated with the Fanconi anemia pathway. Nor has SAN1 to our knowledge been identified as mutated in Fanconi anemia patients. However, the presence of radial chromosomes in response to MMC, a Fanconi-like phenotype, is cause enough to investigate the relationship of SAN1 to the FA pathway. Since our data clearly indicate that SAN1 is not epistatic with the Fanconi anemia pathway, the radial chromosomes and aberrations can be better interpreted as data that support a general defect in the response to ICLs, rather than an involvement of SAN1 in the FA pathway.

Our findings that the loss of SAN1 does not affect FA pathway activation or function are also consistent with an independent role in repair, Mono-ubiquitylation of FANCD2 is a critical step that requires the FA core complex and occurs following lesion recognition (Longerich et al., 2014). Additionally, the functional test of FANCD2 foci formation demonstrates that ICL sites are marked by FANCD2, and that therefore the recruitment of SLX4 and various nucleases can occur (Klein Douwel et al., 2014). These results help to rule out that the sensitivity of SAN1-/- cells to cross-liking agents could be

an off target effect of the loss of SAN1 that impairs FA pathway, and it strongly suggests that SAN1 operates in an alternative repair mechanism.

After determining that SAN1 functions independently of the FA pathway, and that loss of SAN1 does not indirectly affect FA pathway function in ICL repair, we sought to investigate the relationship of SAN1 to other nucleases in ICL repair. For several reasons we believed that one of the strongest candidate nucleases that SAN1 might function with in ICL repair was the nuclease SNM1a. The mechanisms by which SNM1a functions in ICL repair are poorly defined, but it clearly functions independently of the FA pathway (Ishiai et al., 2004). Moreover the relative sensitivities of cells depleted of SNM1a to ICLs in the literature are comparable to what we had observed with SAN1-/- cells (Iyama et al., 2015). Strikingly, our data showed that in addition to similar levels of sensitivity in response to Cisplatin and MMC, that there was no additive decrease in survival upon the loss of both nucleases, suggesting that SAN1 and SNM1a cooperate within the same mechanism to repair ICLs. Since SNM1a has been implicated in both replication dependent and transcription dependent ICL repair processes, it will be important for future work to try and elucidate the specific ICL repair pathway and mechanism in which these proteins cooperate.

The finding that SAN1 is also epistatic to the nuclease FAN1, was more surprising, as FAN1 has a much closer relationship to the Fanconi anemia pathway. However there are clear discrepancies in the literature surrounding whether this relationship to the FA pathway and interaction with FANCD2 is necessary for FAN1 function in ICL repair. Interestingly, the relationship between SNM1a and FAN1 is also controversial. Although we have not investigated the epistatic relationship between these

two nucleases in ICL repair, the depletion of SNM1a has been reported to further increase the sensitivity of FAN1-/- MEFs to cross-linking agents (Thongthip et al., 2016). Although SNM1a has been shown to be non-epistatic to FANCC, indicating it functions outside of the FA pathway, biochemical evidence has complicated this conclusion. *In vitro* studies have instead suggested that XPF and SNM1 cooperate to unhook ICLs in an epistatic manner (Wang et al., 2011).

It is interesting to speculate about how SAN1 might be epistatic and function cooperatively with both SNM1a and FAN1 in ICL repair. Given the biochemical activity of SAN1 on 5' ssDNA and splayed arm substrates, one could imagine a scenario where SAN1 would digest 5' ssDNA substrate to then provide a better dsDNA substrate at a junction near an ICL, thus allowing SNM1a to digest past the cross-linked nucleotides effectively unhooking the lesion. Interestingly, in addition to the 5' flap endonuclease activity that FAN1 displays, recent studies have also demonstrated that FAN1 possesses 5' exonuclease activity that allows it to digest past an ICL similarly to SNM1a (Pizzolato et al., 2015).

However if SNM1a and FAN1 act truly independently of one another as data in MEFs suggests, it is unlikely that all 3 nucleases including SAN1 function together within the same ICL repair mechanism. Instead one possibility is that SAN1 is required to participate in ICL repair with each of these nucleases separately under unique conditions. It is tempting to speculate that this might be a transcription coupled repair mechanism with SNM1a, and given the involvement of FAN1 in the response to replication stress and stalled forks, a replication dependent repair mechanism with FAN1 (Lachaud et al., 2016).

In the future more studies are certainly needed to further explore the relationships between these 5' nucleases implicated in ICL repair, including investigations into their regulation and possible functional differences in various species and biochemical systems.

CHAPTER IV

SAN1 INTERACTS WITH THE RNA:DNA HELICASE SENATAXIN TO FUNCTION IN ICL REPAIR

Abstract

SAN1 has never previously been identified in any large scale screen involving the DNA damage response or interstrand cross-link repair proteins to date. Furthermore, it has never been published as an interacting protein of other components of the DDR machinery. Therefore to investigate further the mechanisms and pathways through which SAN1 might act in the repair of ICLs, we sought to identify potential interacting partners of SAN1. We utilized a yeast two hybrid screening approach and found that SAN1 interacts with the RNA/DNA helicase Senataxin (SETX), which functions in the resolution and removal of RNA:DNA hybrids known as R-loops (Skourti-Stathaki et al., 2011). We have found that this interaction between SAN1 and Senataxin is enhanced in the presence of ICLs, and that it is required for SAN1 function in ICL repair. Moreover SAN1 and Senataxin appear to function in a similar pathway to respond to interstrand cross-links. Finally, we demonstrate that the loss of SAN1 leads to increased R-loop levels, likely as a consequence of a failure to repair interstrand cross-links.

Introduction

Although less well studied than the Fanconi anemia pathway itself, several mechanisms exist for the repair of ICLs that act independently of the FA pathway (Williams et al., 2012) (Williams et al., 2013). To investigate what potential pathways and DNA repair proteins SAN1 might function with outside of the FA pathway, we performed a Y2H screen to identify potential binding partners. The interaction that we believed most likely to inform us about the pathway and mechanism that SAN1 acts in during ICL repair, was with the RNA:DNA helicase Senataxin. Although there were other potentially interesting hits from genes implicated in the DNA damage response, nearly all of these other hits were more likely to be involved in the regulation of SAN1, and included classes of proteins such as kinases and E3 ubiquitin and sumo ligases. Senataxin, also known AOA2 or ALS4, is encoded by the gene SETX and is mutated in both ataxia with oculomotor apraxia type 2 as well as juvenile amyotrophic lateral sclerosis type 4 (Fogel et al., 2006) (Chen et al., 2004). As an RNA:DNA helicase Senataxin plays diverse roles related to RNA metabolism within the DNA damage response in order to maintain genome integrity. Senataxin has been implicated in numerous transcription coupled repair mechanisms through its interaction with the RNA pol II subunit Rrp45, as well as in the response to oxidative stress and in maintaining telomere stability (Suraweera et al., 2007) (De Amicis et al., 2011). Additionally, some studies have suggested an involvement of Senataxin in maintaining circadian rhythms as well as in the cellular response to stressed replication forks (Alzu et al., 2012) (Padmanabhan et al., 2012). The most well-defined function of Senataxin however, is its

role in R-loop regulation, and its function in resolving and unwinding these structures (Skourti-Stathaki et al., 2011).

R-loops are tertiary RNA:DNA structures that form when a nascent mRNA transcript anneals to DNA. The RNA:DNA hybrid strand along with the displaced loop of ssDNA that forms make up the tertiary structure referred to as an R-loop. Although R-loops perform important physiological roles such as in immunoglobulin (Ig) class switching, DNA repair, and regulating gene expression, the dysregulation of R-loop formation, as well as the persistence R-loops, can lead to genomic instability (Skourti-Stathaki et al., 2014). In fact loss of R-loop regulation has been linked to numerous neurological disorders and cancers (Groh and Gromak et al., 2014).

Interestingly, recent evidence in the literature has indicated cross-talk between R-loop resolution pathways and ICL repair mechanisms (Garcia-Rubio et al., 2015) (Schwab et al., 2015). In fact Senataxin has been shown to bind and cooperate with the homologous recombination and ICL repair protein BRCA1, to prevent R-loop accumulation at transcriptional pause sites (Hatchi et al., 2015). Additionally FANCM, a RNA:DNA helicase that is a part of the FA core complex and has been implicated in ICL lesion detection, has been shown to display *in vitro* helicase activity towards R-loop like structures (Schwab et al., 2015). Finally, FANCD2 and other components of the FA pathway have also been implicated in the process of coordinating and preventing transcription replication conflicts, collisions that have been shown to induce R-loop formation (Garcia-Rubio et al., 2015). As R-loops provide a blockade to both transcription and replication complexes similarly to ICLs, some have gone as far to

speculate if they may in fact be one of the central lesions that the FA pathway has evolved to deal with.

In this chapter we explore the relationship between SAN1 and Senataxin in ICL repair, as well as how the function of these proteins in this process relates to R-loop formation. Our studies provide important and novel results that further demonstrate the overlap between pathways involved in interstrand cross-link repair and R-loop resolution.

Results

SAN1 interacts with the RNA:DNA helicase Senataxin

To identify potential interacting partners of SAN1, we had a yeast two hybrid (Y2H) screen performed by Hybrigenics™ using full length mouse SAN1 as bait and a mouse adult brain prey library (Figure 12a). The hit that we believed could be most informative regarding SAN1 function was the RNA/DNA helicase Senataxin, which functions mainly in R-loop resolution. Several overlapping and independent fragments from the screen were shown to potentially interact with SAN1 (Figure 12a). To validate the interaction, we performed a co-immunoprecipitation experiment of the endogenous proteins. In the presence of MMC we were able to successfully Co-IP endogenous SAN1 from HeLa WT cells and detect endogenous Senataxin (Figure 12b), which was not present in the SAN1-/- cell line used as a negative control.

After validating the endogenous interaction of SAN1 and Senataxin, we sought to investigate if the interaction might be important for SAN1 function in ICL repair. To test this hypothesis we utilized a cell line we obtained from Stephen West's lab, that expresses an a SETX-FLAG-GFP construct at near endogenous levels of SETX

expression (Yüce et al., 2013). We then over-expressed the same SAN1-ssf construct as we previously described, and used these cell lines to examine the interaction between SAN1 and Senataxin under varying conditions. We found that when cells were treated with MMC, the interaction between SAN1 and Senataxin function was strongly increased (Figure 12c). This suggests that this interaction might be regulated following ICL induction, and that it might be important SAN1 function in response to ICLs.

Senataxin has a well-established role in various areas of the DNA damage response, including the resolution of R-loops, oxidative DNA damage, and DNA repair at telomeres (Suraweera et al., 2007) (De Amicis et al., 2011). However, little evidence exists of a role for Senataxin in ICL repair. To determine if SETX might play a role in the response to or repair of ICLs, as well as if it might function cooperatively with SAN1, we performed epistasis experiments examining the relationship between these two proteins. We found that depletion of SETX by RNAi in Hela WT cells, sensitized the cells to both Cisplatin and MMC, suggesting some role for SETX in the response to ICLs. More intriguingly, this level of sensitization was similar to that which we observed in the SAN1-/- cells, and there was no further decrease or synergistic effect on cell survival when SETX was depleted in the absence of SAN1 (Figure 12d-f).

Overall these data demonstrate that SAN1 and Senataxin interact endogenously, and that this interaction is enhanced following exposure of cells to MMC. These results combined with the epistasis experiment. suggest that SAN1 and SETX likely cooperate in the response to ICLs, and that the interaction between these two proteins is likely regulated and important for SAN1 function in ICL repair.

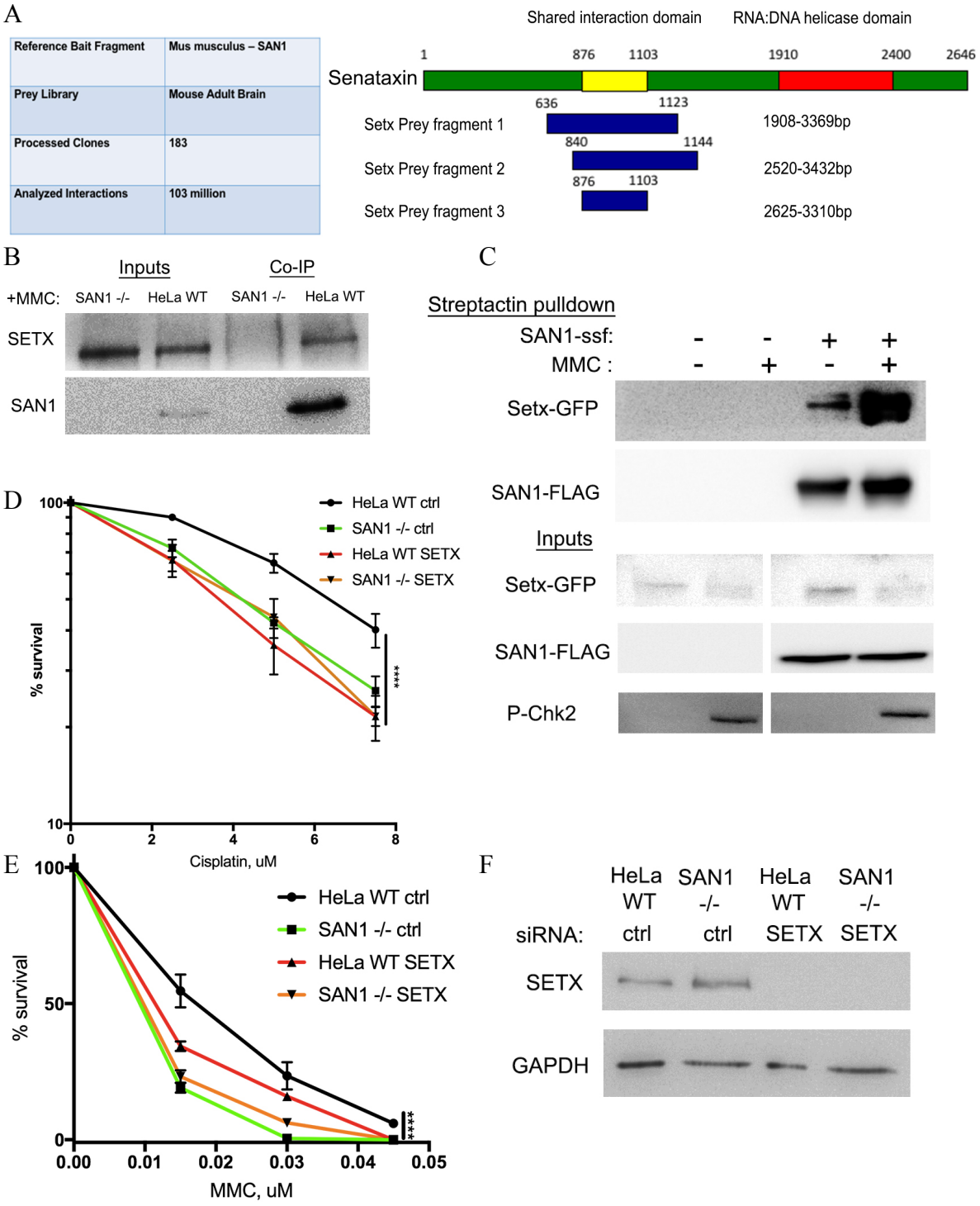


Figure 12. SAN1 interacts with the RNA/DNA helicase Senataxin.
(a) Yeast two-hybrid (Y2H) screen and library information in addition to a schematic detailing mSETX prey fragments that interacted with mSAN1 in Y2H screen. The shared interaction domain of mSETX prey fragments shows that SAN1 interacts with SETX in a

region of the N-terminus and not near the RNA/DNA helicase domain at the C-terminus. (b) Endogenous SAN1 was co-immunoprecipitated from HeLa WT and SAN1 -/- after treatment with 1 μM MMC. Top panel: immunoblot of Senataxin inputs (lanes 1-2) and Co-IP (lanes 3-4), bottom panel: immunoblot of SAN1 inputs (2%) (lanes 1-2), and Co-IP (lanes 3-4). (c) A stable HeLa cell line expressing near endogenous levels of a Senataxin-FLAG-GFP construct was transduced with a lentiviral construct of SAN1WT-$Strep_2$-FLAG (SAN1ssf). Soluble nuclear fraction was isolated from the cells and SAN1 was captured on Strep-Tactin beads.. Top Panel: Immunoblots for Senataxin and SAN1 of precipitations from HeLa SETX-FLAG-GFP cell line +/- SAN1-ssf and +/- MMC. Bottom panel: Input immunoblots for Senataxin ,SAN1 and P-Chk2 from HeLa SETX-FLAG-GFP cell lines +/- SAN1-ssf, and +/- 1μM MMC . (d-e) CSAs of HeLa WT and SAN1-/- cells, transfected with scrambled ctrl or SETX siRNAs, in response to Cisplatin and MMC. Statistical significance determined by two-way ANOVA. Error bars denote s.e.m. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. MMC CSA is shown in linear scale in panel (e) due to zero values at higher MMC concentrations. (f) Immunoblot of SETX siRNA knockdown.

The SAN1-SETX interaction is required for SAN1 function

To further explore the relationship between Senataxin, SAN1, and ICL repair, we performed mapping experiments to identify the region of SAN1 that is required for the interaction with Senataxin. We created a Myc tagged SETX fragment construct, generated from the shared interaction domain of the SETX hits from the yeast two hybrid screen (Myc-mSETXSID). We then performed Co-IP experiments after over-expressing Myc-mSETXSID and human SAN1WT-FLAG or SAN1Δrepeats-FLAG constructs in human embryonic kidney (HEK) 293 cells. We found that the human SAN1WT was able to interact with the mouse Myc-mSETXSID fragment, however the SAN1 construct lacking the repeats region between the N-terminal nuclease domain and the C-terminus of the protein (SAN1Δrepeats), was unable to do so (Figure 13a). The flexible repeat region consists of ~200 amino acids and is predicted to be largely unstructured. The number of 10-12 semi-conserved repeating amino acids varies from species to species, with most higher order organisms containing a larger number of repeats. We speculate that this

region might acts a flexible linker that allows the C-terminal region of SAN1 to interact with the N-terminal nuclease domain, as well as serving as a potential site for protein-protein interactions such as with Senataxin.

We next tested if full length Senataxin was unable to interact with SAN1WT-ssf or SAN1Δrepeats-ssf by over-expressing these constructs in the HeLa SETX-GFP-FLAG cell line previously described. Similarly to the initial mapping data with Myc-mSETXSID, we found that SAN1WT but not SAN1Δrepeats was able to interact with full length human Senataxin, confirming our initial mapping data (Figure 13b). Because the SAN1Δrepeats lacked the region required for an interaction with Senataxin, we next tested if this mutant still retained its nuclease activity, as it could potentially allow for us to definitively test if this interaction is necessary for SAN1function in ICL repair. We found that unlike the catalytically inactive SAN1D90A mutant, the SAN1Δrepeats mutant displayed nuclease activity on ssDNA at levels comparable to the WT protein (Figure 13e). Since the SAN1Δrepeats mutant lacked the ability to interact with SETX, but retained its nuclease activity, we asked if it was able to restore resistance to cross-linking agents in SAN1-/- cells. Strikingly, we found that mutant was completely unable to do so (Figure 13c-d). Collectively, these data demonstrate that SAN1 and SETX function together in ICL repair, and that the interaction of SAN1 with Senataxin is required for SAN1 function in response to ICLs.

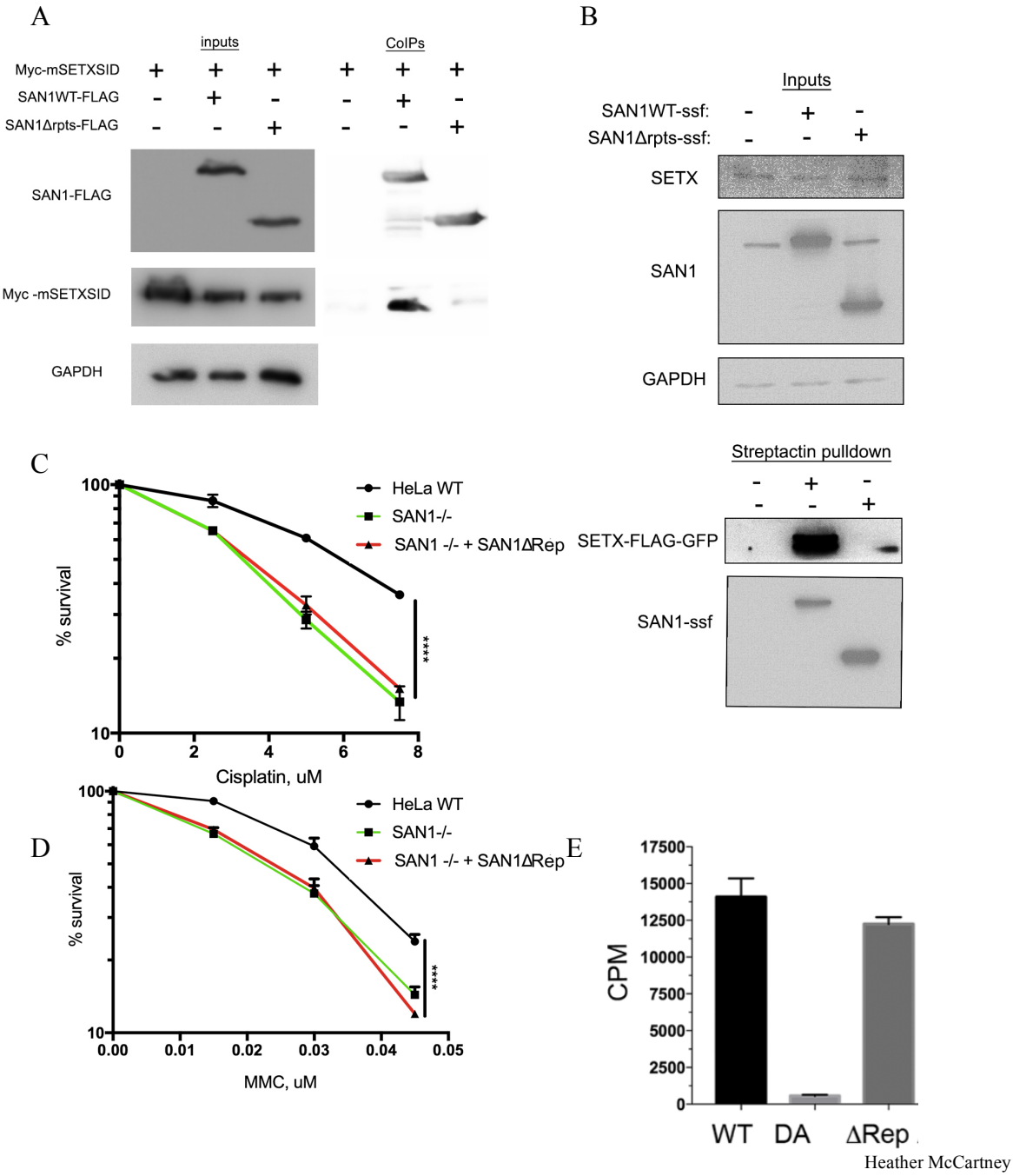


Figure 13. The SAN1/Senataxin interaction is required for SAN1 function in ICL repair.

(a) Mapping of the binding region of hSAN1 to the shared interaction domain (SID) of mSETX determined by the Y2H library fragments. Cells were transfected with myc-mSETXSID and either empty vector, SAN1WT-FLAG, or SAN1ΔRep-FLAG. SAN1WT-FLAG or SAN1ΔRep-FLAG were co-immunoprecipitated and lysates were blotted for FLAG and Myc. (b) Cells were fractionated to prepare the soluble nuclear

fraction and SAN1 was captured on Strep-Tactin beads. Upper panel: immunoblots of inputs for stable HeLa cell lines expressing near endogenous levels of a Senataxin-FLAG-GFP construct and over-expressing SAN1WT-Strep$_2$-FLAG (SAN1ssf) or SAN1 lacking the central repeats region (SAN1ΔRep-ssf). Lower panel: co-immunoprecipation of SETX-FLAG with SAN1WT-ssf but not SAN1ΔRep-ssf. (c-d) CSAs for HeLa WT, SAN1-/-, and SAN1-/- +SAN1ΔRep-ssf cells exposed to Cisplatin and MMC. Statistical significance determined by two-way ANOVA. Statistical significance determined by two-way ANOVA. Error bars denote s.e.m. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. Schematic of SAN1 deletion mutants used in f, g, h followed by immunoblot of WT, DA, and ΔRepeats proteins purified from 293T cells; and (i) tested for nuclease activity against 5' ^{32}P labeled ssDNA using the filter spin assay (N=2, error bars show range).

As a RNA/DNA helicase, a central function of Senataxin lies in transcription coupled repair mechanisms and the resolution of R-loops. Interestingly, R-loop resolution has recently been linked to the FA pathway (Schwab et al., 2015) (Garcia-Rubio et al., 2015), and to BRCA1 (Hatchi et al., 2015), a protein essential for homologous recombination and resistance to ICLs. Indeed, BRCA1 binds directly to SETX in an association that is required for DNA damage repair at transcriptional pause sites (Hatchi et al., 2015). Given the importance of the interaction with Senataxin for SAN1 function in ICL repair, we aimed to investigate the relationships between R-loops, ICL repair, and the functions of SAN1 and Senataxin.

R-loops formation occurs naturally during transcription stalling and pausing, and can be induced by collisions between the replication and transcription machinery, negative super-coiling of DNA, or regions of G-quartets (Santos-Pereira et al., 2015). In fact some evidence exists that chemotherapeutic drugs which block transcription and cause stalling of RNA pol II, such as Camptothecin and MMC, can also lead to R-loop formation (Marinello et al., 2016) (Garcia-Rubio et al., 2015).

In addition to providing a blockade for biological processes such as DNA replication and transcription, persistent R-loops are a potential source of DNA damage and genomic instability. R-loops that are not properly resolved have shown to be processed by nucleotide excision repair nucleases into double strand breaks (Sollier et al., 2014).

Loss of SAN1 causes increased R-loops in response to MMC

We first considered the possibility that SAN1-/- cells might display elevated levels of R-loops following exposure to MMC. To investigate this hypothesis, we utilized a monoclonal antibody (S9.6) that can specifically detect RNA:DNA hybrids (Sridhara et al., 2017). We examined R-loop levels by immunofluorescence in both HeLa WT and SAN1-/- cells treated with vehicle or MMC (Figure 14a). Because the S9.6 antibody displays high background signal in the cytoplasm, as well as high signal in nucleolar regions that contain many types of RNAs, we co-stained with DRAQ5 and an anti nucleolin antibody. The intensity of the S9.6 signal labeling R-loops was then measured from the nuclear regions after creating a DRAQ5 mask to subtract the cytoplasm, and a nucleolin mask to exclude these areas. As clearly shown in Figure 14c, we observed an strong increase in the levels of R-loops present in SAN1-/- cells compared to HeLa WT cells after MMC treatment. We speculate that this large increase in R-loop levels results from unrepaired ICLs that exist in SAN1-/- cells treated with MMC, which results in more frequent transcriptional stalling and R-loop formation. This increase in quantified as the mean fluorescent intensity of R-loop staining from three independent experiments (Figure 14b). Interestingly, we observe a smaller but slightly more significant increase in

R-loop levels in SAN1-/- compared to HeLa WT cells in untreated conditions (Figure 14b). This might be the result of cross-links that exist in untreated conditions from endogenous sources such as aldehydes that also cause transcriptional blockage (Price et al., 2014). Worth noting however is the clear difference in how WT and SAN1-/- cells respond to MMC treatment in this assay. While WT cells show no significant change in R-loop levels after MMC treatment, the SAN1-/- exhibit much higher R-loop levels in response to MMC compared to treatment with vehicle (Figure 14a-c).

Although we cannot rule out that SAN1 might play some role in R-loop resolution, we believe our data support a model where R-loops arise indirectly as a consequence of the failure to repair and remove ICLs. This model is consistent with the striking difference in how R-loop levels change in WT and SAN1-/- cells after MMC treatment. Additionally, although some of the DNA damage in SAN1-/- cells after MMC treatment might be attributable to increased R-loop levels, failure to repair or remove R-loops has never been previously shown to cause radial chromosomes or chromosomal aberrations. Therefore, it is highly likely that these radial chromosomes and chromosomal aberrations (Figure 5) indicate a more direct involvement of SAN1 in ICL repair, and not a role in general R-loop resolution with Senataxin.

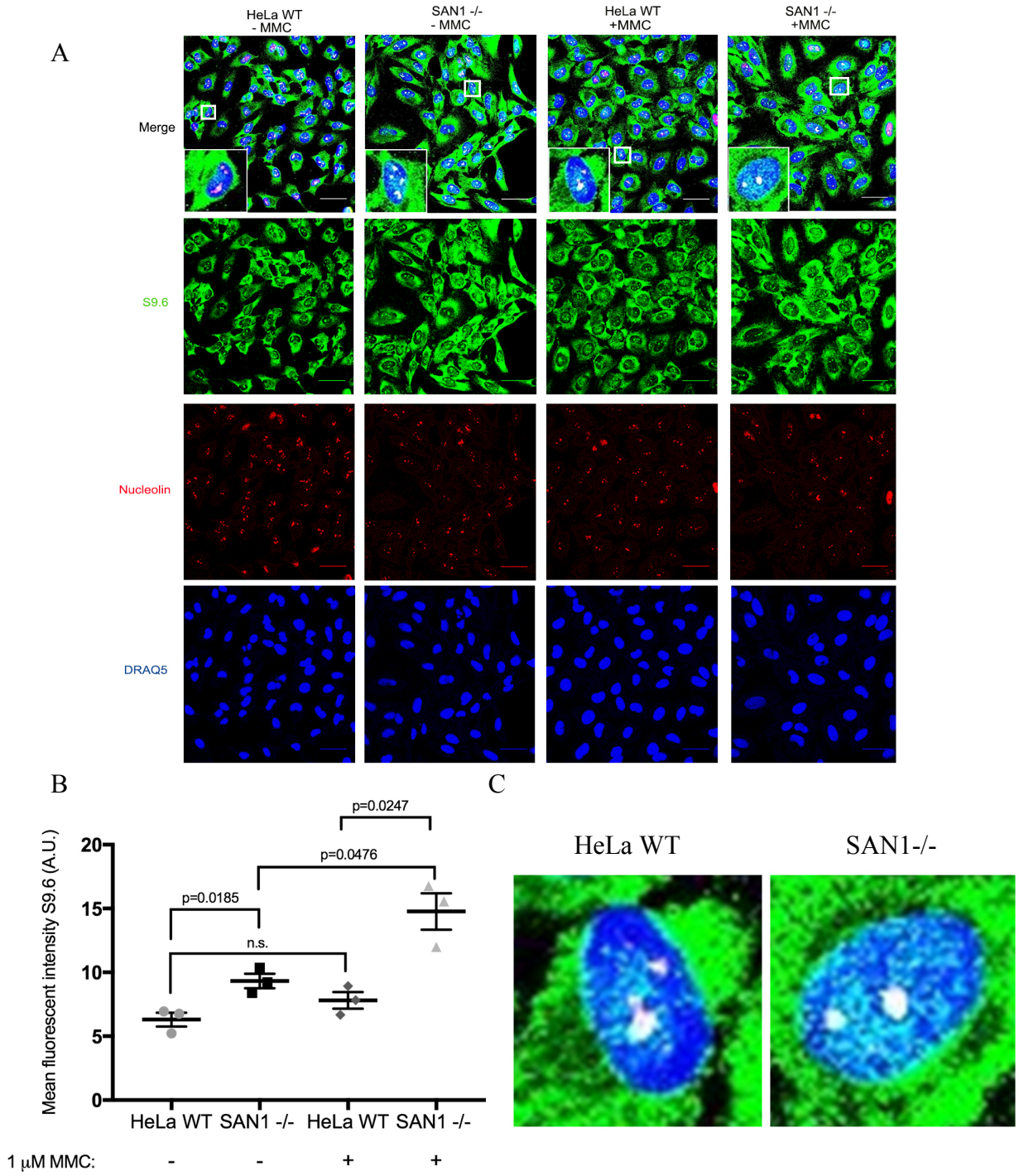
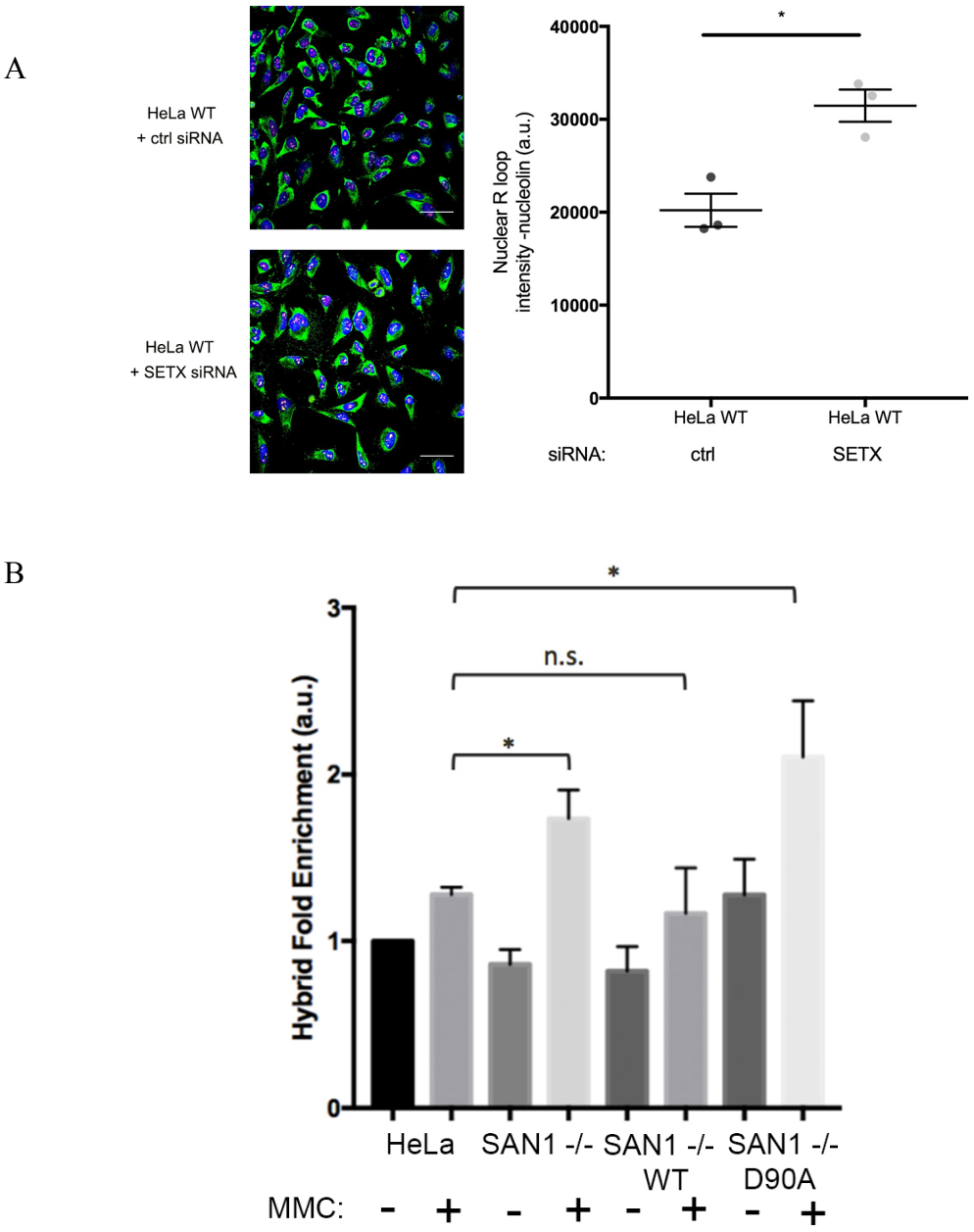


Figure 14. SAN1-/- cells display increased levels of R-loops in response to MMC. (a) HeLa WT and SAN1-/- cells were treated with vehicle or 1 μM MMC and labeled with a monoclonal antibody to detect R-loops (S9.6), a polyclonal antibody against nucleolin, and Draq5 to label DNA. Intensity of nuclear R-loop staining was quantified from the nucleus following masking with the DRAQ5 channel, and subtraction from nucleolin regions. (b) Quantification of nuclear R-loop intensity from 3 independent

experiments. Statistical significance was calculated using unpaired t-test (N=3 biological replicates, at least 60 cells per sample were analyzed). (c) Enlarged images of HeLa WT and SAN1-/- MMC treated cells showing elevated R-loop staining.

To validate the R-loop staining results, we transfected HeLa WT cells with ctrl scrambled or a SETX siRNAs and examined R-loop levels as a positive control. Similarly to data that has been previously reported, depletion of Senataxin resulted in a significant increase in the levels of R-loops (Figure 15a) (Skourti-Stathaki et al., 2011).

As an additional independent approach to examine the relationship between SAN1, R-loops, and ICL repair, we also utilized a slot blot assay to examine R-loop levels in SAN1-/- cells and rescue lines. In this assay cells were treated with vehicle or MMC, and whole genomic material was isolated and spotted on a membrane. The material was then probed for R-loops using the S9.6 antibody, and for ssDNA as a loading control. We observed that in the presence of ICLs, SAN1-/- cells that had been treated with MMC showed increased R-loop levels compared to the HeLa WT cells (Figure 15b) (Heather McCartney). The elevated R-loop levels were reduced to levels comparable to HeLa WT cells upon over-expression of SAN1WT, but not after over-expression of SAN1D90A mutant, indicating that the nuclease activity of SAN1 is required to prevent this. Interestingly, we did not observe an increase in R-loops in untreated conditions in SAN1-/- cells compared to the HeLa WT cells as observed in the immunofluorescence experiments. This difference might be attributable to inherent differences and sensitivities between the two assays.



Heather McCartney

Figure 15. Specificity of S9.6 staining and Slot blot analysis
(A) Left panel: Immunofluorescence images of HeLa WT cells transfected with ctrl or SETX siRNA and stained with a monoclonal antibody to detect R-loops (S9.6), a polyclonal antibody against nucleolin, and Draq5 to label DNA. Right panel: Quantification of R-loop intensity. Statistical significance determined by t-test with Welch's correction (N=3 biological replicates, at least 70 cells were analyzed per sample). (b) Validation of S9.6 R-loop antibody specificity by pre-treatment with R-loop degrading enzyme RNAse H in HeLa WT and SAN1-/- cells treated with 1 μM MMC. (c) Dot blot assay for quantification of RNA/DNA hybrids. Total genomic DNA on a

charged nylon membrane was cross-linked with UV, and probed with the mouse S9.6 antibody. Spot density was normalized to denatured DNA probed with antibody against ssDNA. (N=4) Statistical significance was determined by using unpaired t-test to compare each condition to HeLa treated.

Additionally, to validate the specificity of the S9.6 antibody we pre-treated cells with RNase H following fixation and permeabilization. RNase H is a ribonuclease capable of degrading R-loops in nucleolytic manner (Amon et al., 2016). As predicted RNase H completely abolished signal of the S9.6 antibody thereby validating the specificity of the antibody against RNA:DNA hybrids (Figure 16a).

A

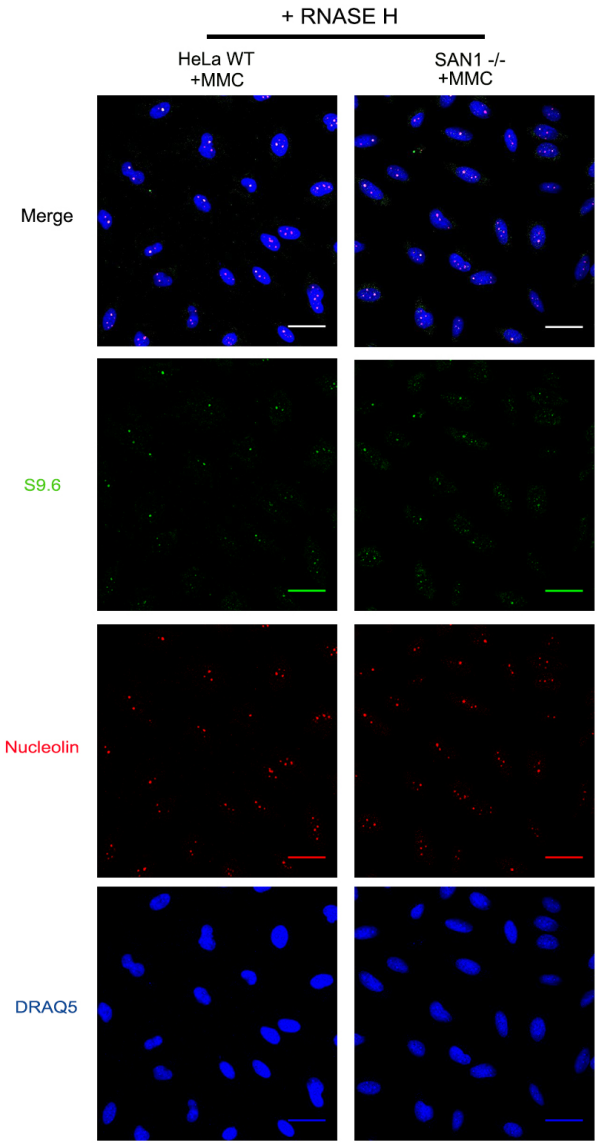


Figure 16. Validation of S9.6 R-loop staining by RNAse H treatment.
(a) Validation of S9.6 R-loop antibody specificity by pre-treatment with R-loop degrading enzyme RNAse H in HeLa WT and SAN1-/- cells treated with 1 μM MMC.

Potential involvement of SAN1 in transcription coupled ICL repair

Given the involvement of Senataxin in R-loop resolution, a transcriptionally coupled repair process, we hypothesized that SAN1 might also function in a transcriptionally coupled repair mechanism. This possibility seemed plausible as SAN1 clearly functions independently of the Fanconi anemia ICL repair pathway, a replication-dependent repair mechanism for interstrand cross-links. Moreover, the increase in R-loops and required interaction of SAN1 with SETX to function in ICL repair provided further evidence for this potential repair mechanism. We hypothesized that some of the DNA damage present in SAN1-/- cells, shown by increased γH2AX intensity and 53BP1 foci (Figure 6, Figure 7), might be a result of transcription-associated damage from R-loop formation.

To examine this possibility we treated HeLa WT and SAN1 -/- cells with MMC and vehicle or the transcriptional inhibitor cordycepin, as has been previously described (Garcia-Rubio et al., 2015; Schwab et al., 2015). Strikingly, treatment with cordycepin decreased γH2AX in MMC-treated SAN1-/- cells to levels comparable to WT cells (Figures 17a-b). These data indicate that the increased DNA damage induced by ICLs in SAN1-/- cells is transcription dependent. However an important caveat to this experiment is that cordycepin, an Adenosine analog that inhibits transcriptional elongation, has numerous off target effects (Tuli et al., 2013). Additionally, we stained the cells treated with MMC in under these conditions using Bromodeoxyuridine (BRDU), a Thymidine analog that is actively incorporated into DNA during replication (data not shown) (Leet et al., 2006). We found that treatment of cells with 1 μM MMC for 30 hours, a condition in which we see a strong increase in γH2AX by immunofluorescence, resulted in a

significant reduction in BRDU incorporation. This suggests that both HeLa WT and SAN1-/- cells are likely arresting at this concentration of MMC for this time period, making it difficult to distinguish if the associated DNA damage caused by ICLs in SAN1-/- cells is dependent on transcription or replication.

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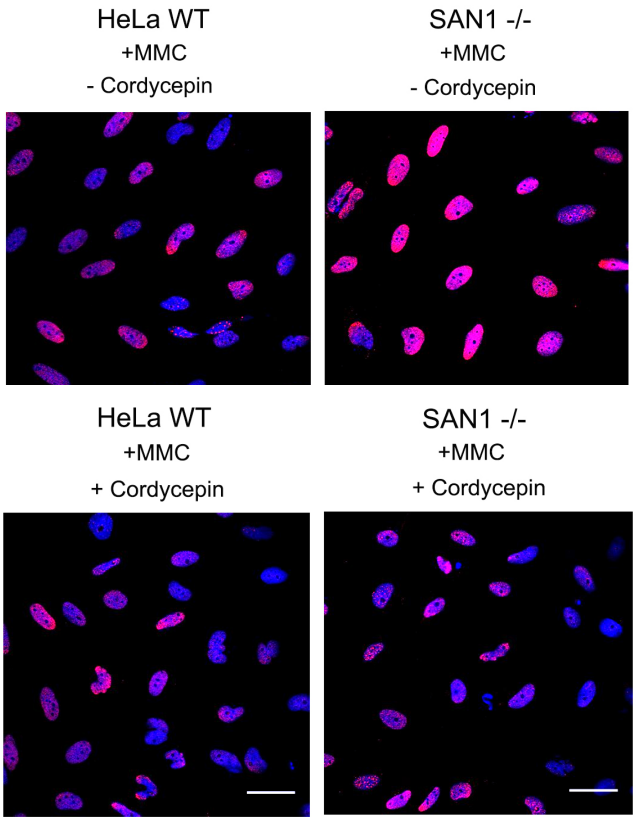

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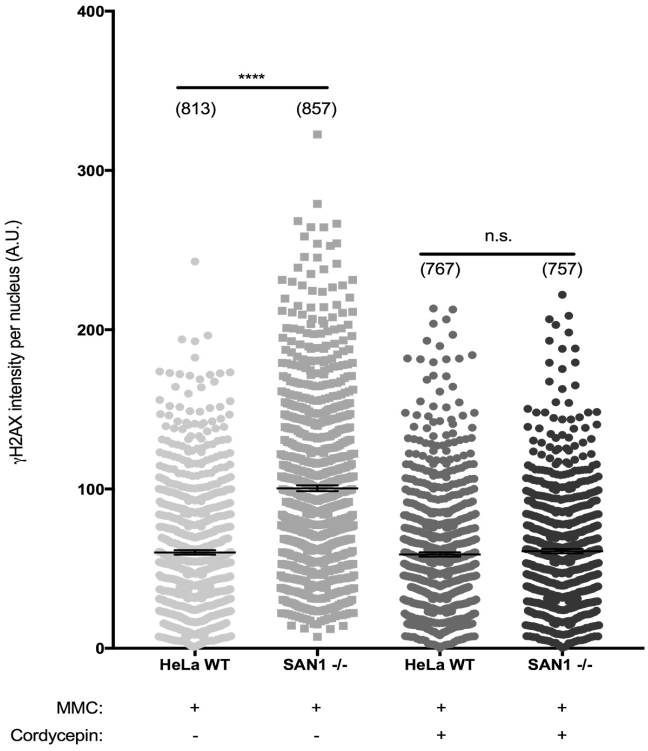

Figure 17. Inhibition of transcription by Cordycepin prevents ICL associated DNA damage. (a) Immunofluorescence images of γH2AX in HeLa WT and SAN1-/- cells

treated with vehicle or 1 μM MMC and 75 uM Cordycepin. (b) Quantification of γH2AX in HeLa WT and SAN1-/- cells treated with vehicle or 1 μM MMC and 75 uM Cordycepin (N=3). Statistical significance calculated using Mann-Whitney U test.

Due to these concerns with the Cordycepin experiments, we performed an additional approach where we treated HeLa WT and SAN1-/- cells with MMC and the RNA pol II inhibitor α-amanitin for 6 hours, and examined the percentage of γH2AX positive cells (Figure 18a-b) (Lee et al., 2008). There was a small increase in the percentage of γH2AX positive cells in SAN1-/- cells compared to HeLa WT, but the phenotype was much less striking than the strong increase in γH2AX intensity observed when the cells were treated for a longer time period with MMC (Figure 17). However, α-amanitin decreased the percentage of γH2AX positive cells for both HeLa WT and SAN1-/- cells in the presence of MMC (Figure 18a-b).

Based on these results we are unable to conclude if the SAN1 functions specifically in a transcription-coupled repair or some other replication-independent repair process in response to ICLs. This remains an important area of research that will provide valuable insight into how SAN1 functions in ICL repair, and will need to be investigated further in future studies.

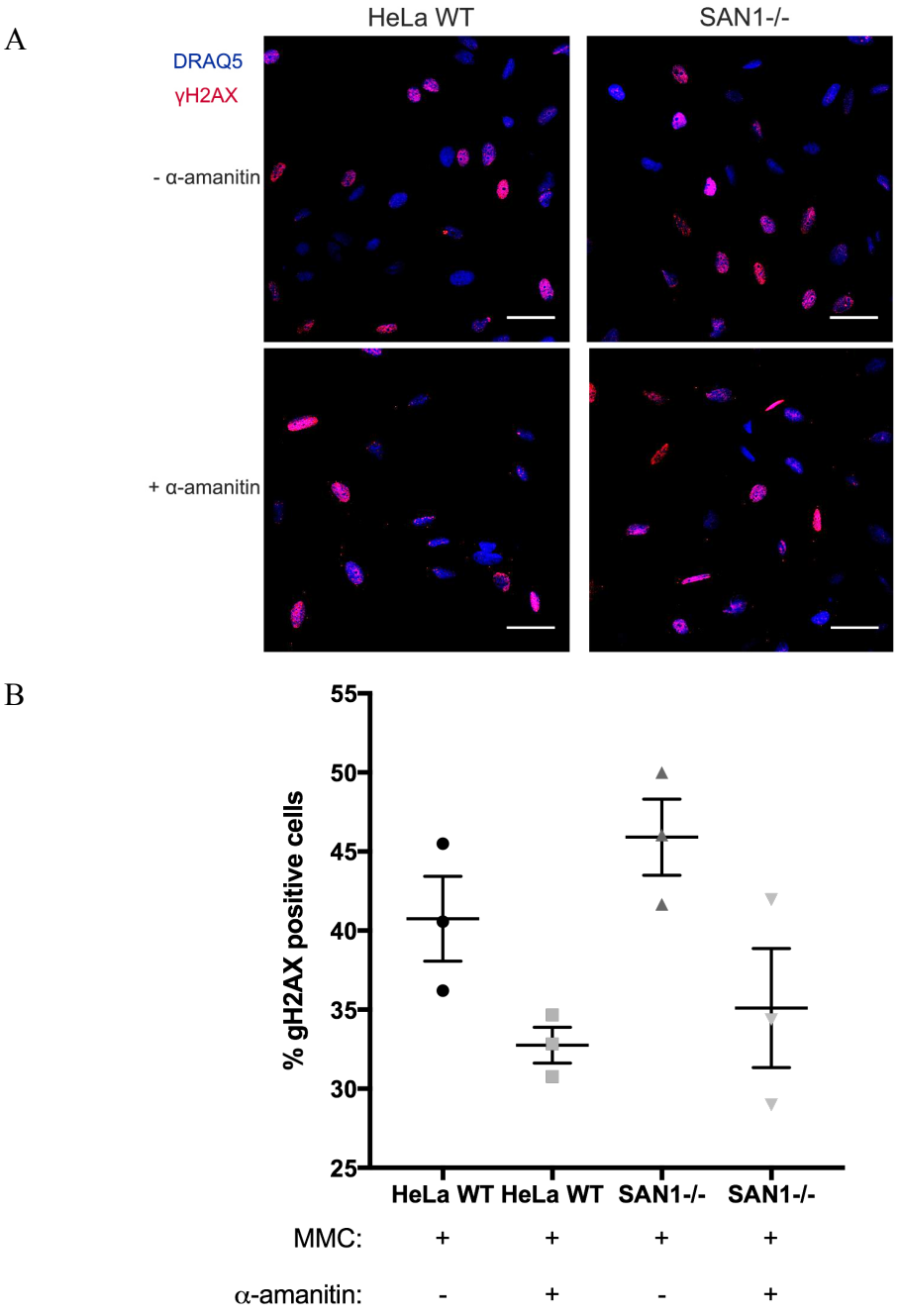

Figure 18. DNA damage after inhibition of transcription by α-amanitin is reduced
(a) Immunofluorescence images of cells treated with 10 μM MMC and vehicle or 50μg/mL of α-amanitin for 6 hrs to selectively inhibit RNA pol II, then stained for γH2AX and DNA (DRAQ). (b) Quantification of data in (a) measuring the percentage of cells positive for γH2AX staining (N=3 biological replicates, at least 200 cells per sample were analyzed).

The relationship between SAN1, R-loops, and ICLs

To further probe the relationship between SAN1, R-loops, and ICLs, we over-expressed a RNaseH1-NLS-mCherry construct to degrade R-loops in the nucleus, and examined the response to MMC in HeLa WT and SAN1-/- cells (Britton et al., 2014). Over-expression of RNaseH1 caused an increase in γH2AX intensity in response to MMC in HeLa WT cells, while γH2AX in SAN1-/- cells was reduced to levels comparable to the WT cells (Figure 19a-b). We speculate that the formation of R-loops adjacent to ICLs in HeLa WT cells might be necessary for SAN1 to participate in a repair process required to prevent or repair DNA damage, possibly through the recruitment of SAN1 through its interaction with Senataxin (Figure 19). Additionally, the reduction of γH2AX in MMC treated SAN1-/- cells over-expressing RNaseH1, might result from of the removal R-loops that contribute to DNA damage as a consequence of unrepaired ICLs.

Figure 19

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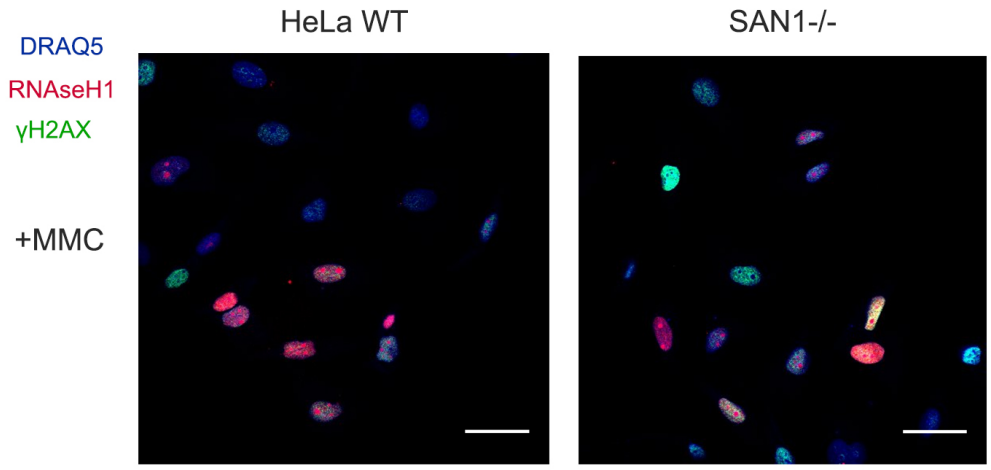

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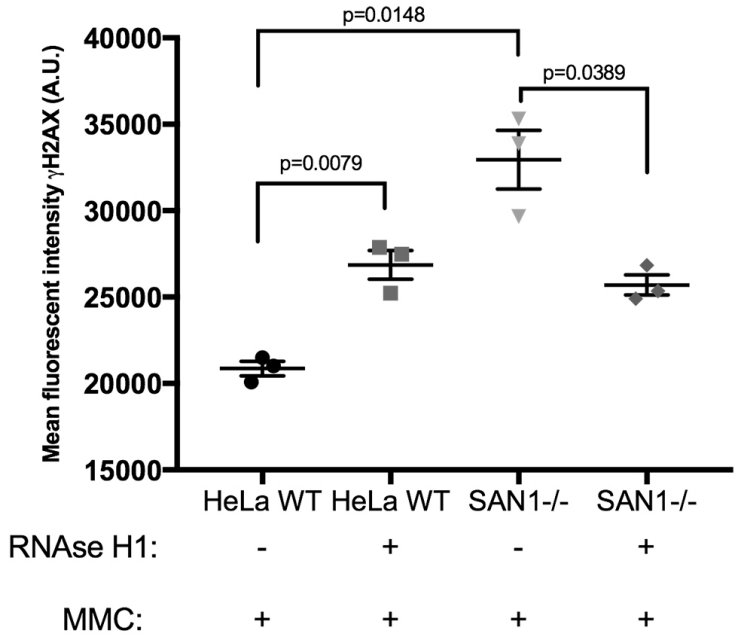

Figure 19. Removal of R-loops by RNaseH1 reduces DNA damage in SAN1-/- cells. (a) Representative immunofluorescence image of cells expressing RNaseH1-NLS-mCherry and treated with 1μM MMC for 30 hrs, then stained for γH2AX and DNA. (b) Quantification of data in (a) measuring the mean fluorescent intensity of γH2AX in cells positive and negative for RNaseH1-NLS-mCherry (N=3 biological replicates, at least 60 cells per sample were analyzed). Statistical significance determined by t-test with Welch's correction.

This data is somewhat difficult to interpret, given the complex relationship that likely exists between SAN1 and R-loops in ICL repair. However these data do fit with our speculative model of the function of SAN1 in ICL repair, as shown in Figure 20.

Discussion

Overlap between cross-link repair pathways such as the Fanconi anemia pathway and R-loop resolution pathways has been highlighted in the literature in recent years (Garcia-Rubio et al., 2015) (Schwab et al., 2015) (Hatchi et al., 2015). The data provided here show an additional previously uncharacterized link between ICL repair and R-loop resolution, through the helicase Senataxin and the newly identified nuclease SAN1. Our data indicate that SAN1 and Senataxin are critical for the cellular response to ICLs and the prevention of R-loop accumulation. Although we cannot definitively say that SAN1 plays no direct role in R-loop resolution, we believe our data strongly supports a model in which R-loops arise as a downstream consequence of unrepaired ICLs and therefore increased incidences of transcription stalling. One alternative possibility however, is that SAN1 might influence the activity of Senataxin in R-loop resolution.

Given that our data show an enhanced interaction between SAN1 and SETX following ICL induction, as well as the requirement for SAN1 to bind SETX to participate in ICL repair, it is tempting to speculate that Senataxin might act as a mode of recruitment of SAN1 to ICL sites. This could occur plausibly through SAN1 binding to Senataxin following its action of unwinding and resolving a R-loop that has formed near the ICL (Figure 20). Following re-annealing of the ssDNA portion of the tertiary RNA:DNA hybrid structure, this would potentially result in a splayed arm structure

containing a 5' ssDNA flap, a substrate SAN1 has shown significant nuclease activity towards. The role of SAN1 might then be to digest this 5' ssDNA down towards the ssDNA/dsDNA junction near the ICL, providing a potentially better substrate for the nucleases SNM1a or FAN1 to digest past the ICL in a step similar to unhooking of the lesion.

One possible reason that cells might require this alternative ICL repair mechanism to the Fanconi anemia pathway, could be due to the steric hindrance that stalled transcription complexes and R-loops create. Strong evidence exists that the Fanconi anemia pathway requires dual convergence of replication forks in order to activate the pathway and repair ICLs (Zhang et al., 2015). In the speculative model shown in Figure 20 below, a replication fork might theoretically arrive from the right side, however a converging fork would not be able to reach an ICL from the left side given the blockage that a stalled RNA pol II enzyme and R-loop would present. Future studies that investigate the overlap between these repair pathways, as well as those that study the role of SAN1 and other nucleases in ICL repair will be critical for our overall understanding of the DNA damage response.

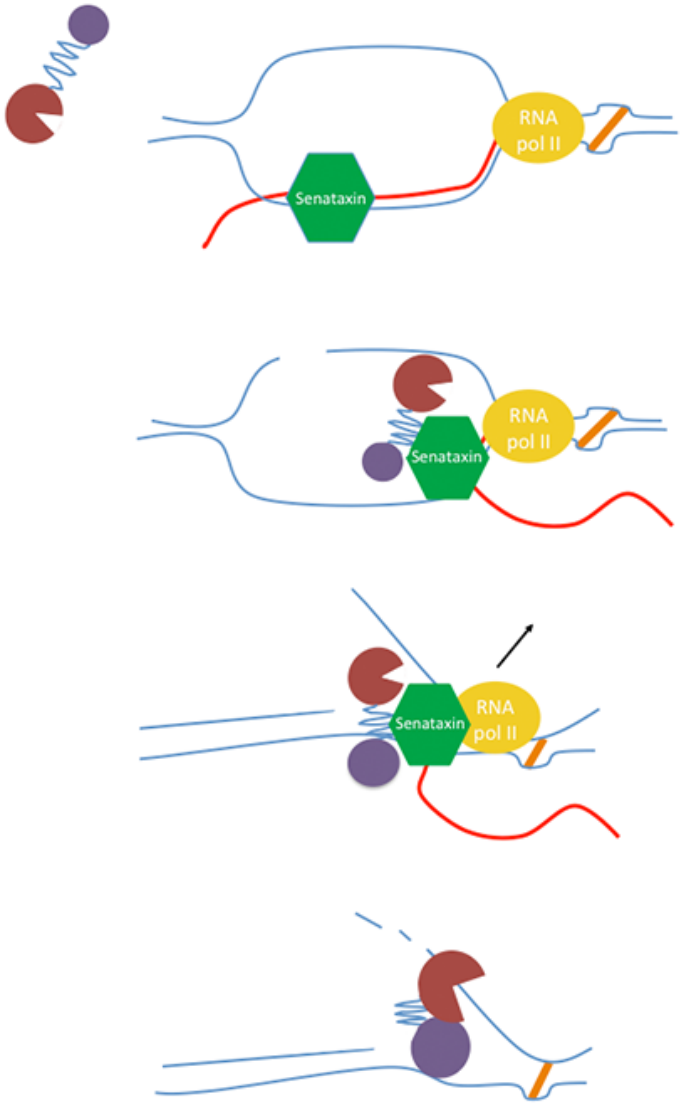

Figure 20. Speculative model for SAN1 function with SETX in processing of ICLs. Collision of transcription complexes with an ICL results in the formation of R-loops. The helicase SETX is recruited to the RNA/DNA hybrid and unwinds it. SETX also recruits the SAN1 nuclease. If an incision is generated in the ssDNA loop, perhaps by XPF, SAN1 can digest the free 5' end back to the ICL, where other nucleases participate in unhooking of the lesion.

CHAPTER V

ADDITIONAL STUDIES

A better understanding of the physiological response to ICLs, including the repair pathways and nucleases required to prevent cross-link hypersensitivity, has important implications for human health and chemotherapeutic treatments. In addition to investigating the molecular functions of SAN1 in human cells, we also carried out preliminary studies to investigate if SAN1 plays an important role in the physiological response to cross-linking agents in mice. In general the DNA repair pathways in mice responsible for the response to cross-linking agents differ significantly in comparison to humans. Strong evidence of this is displayed in the relatively mild phenotypes that mice exhibit upon loss of many of the components of the Fanconi anemia pathway (Parmar, D'Andrea, and Niedernhofer et al., 2009). However the reasons for this are still debated (Sumpter et al., 2016), and some mouse models that inactivate downstream components of the pathway such as SLX4 display much more severe ICL sensitivities and Fanconi like phenotypes (Crossan et al., 2011).

Sensitivities of mice that lack ICL repair nucleases vary widely. For example, FAN1-/- mice display strong sensitivities to cross-linking agents, but like humans also do not have Fanconi anemia like phenotype and instead present with severe kidney abnormalities (Thongthip et al., 2016) (Lachaud et al., 2016). In contrast, a knockout mouse model of SNM1a displays only mild sensitivity to ICLs, but the mice are in general fertile and viable with no other clear phenotype (Dronkert et al., 2000).

Sensitivity of SAN1-/- mice to cross-linking agents

To investigate if SAN1-/- mice might also display sensitivity to ICL inducing agents, we utilized a fam120b-/- mouse model that was generated as previously described (Skames et al., 2011). Preliminary data clearly showed a strong sensitivity of immortalized SAN1-/- MEFs in comparison to SAN1+/+ cells to cross-linking agents in colony survival assays. To examine potential sensitivity of whole animals to MMC, our breeding scheme dictated that we would need to compare heterozygous SAN1+/- mice to matched homozygous SAN1-/- littermates. As a precaution to ensure that there was not too severe of a loss of function of SAN+/- mice or cells to cross-linking agents we first immortalized and tested heterozygous MEFs in comparison to SAN1-/- MEFs in response to both Cisplatin and MMC (Figure 21a-b). Similarly to results from the SAN1+/+ and -/- MEFs shown in Figure 4, we observed a significant decrease in survival of the SAN1-/- compared to cells generated from heterozygous littermates (Figure 21a-b).

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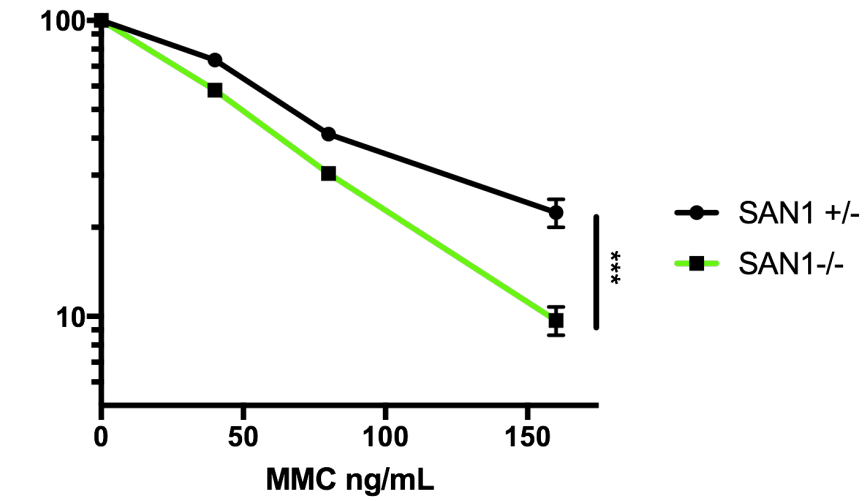

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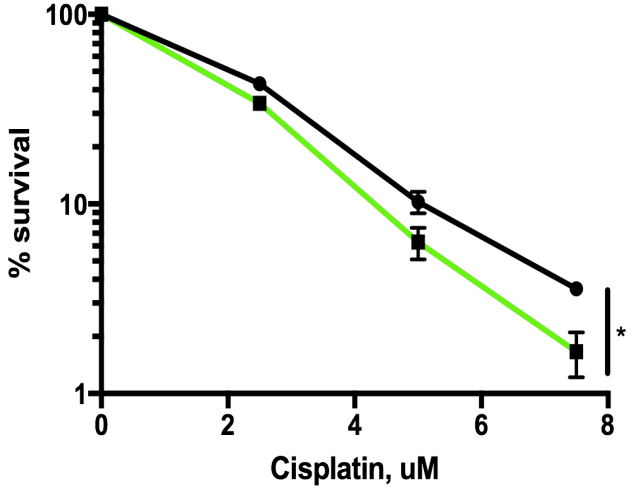

Figure 21. Sensitivity of SAN +/- and -/- MEFs to cross-linking agents. (a-b) Mouse embryonic fibroblasts (MEFs) were generated and immortalized using large SV40 T antigen. Colony survival assays between SAN1 heterozygous homozygous MEFs were performed as described in Figure 4 in response to Cisplatin and MMC. Statistcal significance was determined by two way ANOVA test,

We were then able to address the question of the potential physiological role of SAN1 in response to ICLs in mice. SAN1+/- and -/- mice were injected with 10 mg/kg of MMC intraperitoneally, and monitored over the course of 21 days as has been previously described (Thongthip et al., 2016) (Figure 22a). In sharp contrast to similar experiments carried out in FAN1-/- mice, we did not see an immediate drop in body weight of the SAN1-/- mice (data not shown) (Thongthip et al., 2016). Instead mice that succumbed to the MMC injection and had to be sacrificed displayed a lethargic and weakness phenotype, the cause of which remains unclear. Overall the SAN1-/- mice displayed very mild sensitivity compared to their heterozygous littermates. A phenotype somewhat similar to that found in experiments using a knockout SNM1a mouse model (Dronkert et al., 2000). The relatively mild phenotype might be attributable to the out-bred strain of mice used for these experiments, where variations between the backgrounds of litter mates could contribute to different physiological responses to ICLs, possibly masking some of the ICL sensitivity phenotype we observed. In future studies it will be important to determine if the mild sensitivity to MMC observed in these mice is definitive, as well as to investigate the potential physiological cause of this sensitivity to cross-linking agents.

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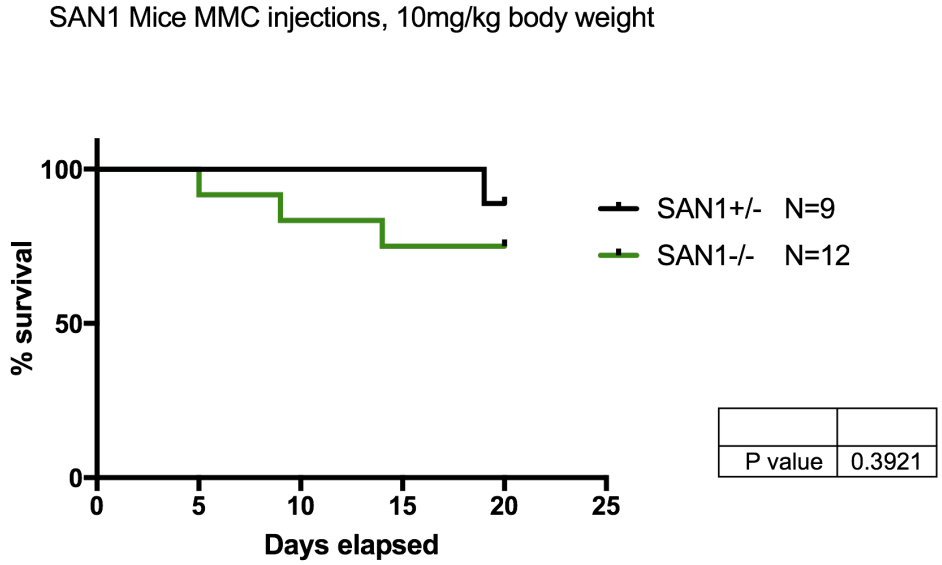

Figure 22. Sensitivity of SAN1-/- mice to Mitomycin C. (a) SAN1 heterozygous and homozygous matched littermates were injected intraperitoneally with 10mg/kg MMC and monitored for survival and weight loss over the the course of 21 days. Statistical significance determined by Kaplan-Meier log rank test.

SAN1 interacts with the E3 SUMO ligase PIAS1

In addition to the identification of Senataxin as an interacting protein in the yeast two hybrid (Y2H) screen, multiple other hits of potential interacting proteins have been implicated in the DNA damage response. Many of them have potential implications for the possible regulation of SAN1 function in ICL repair. Of particular interest is the hit for an E3 SUMO ligase, protein inhibitor of activated STAT 1 (PIAS1). PIAS1 and the related gene PIAS4 have been implicated in regulating the response to double strand breaks through their SUMO ligase activities (Galanty et al. 2009). Furthermore, PIAS1 has been shown to interact with SNM1a, an interaction that was found to be required for proper localization of the nuclease to nuclear foci (Ishiai et al., 2004). Given this Y2H hit

and the epistatic relationship of SAN1 and SNM1a in response to cross-linking agents, we asked if SAN1 and PIAS1 also interact. In 293T cells we over-expressed Myc-mPIAS1 and mSAN1-FLAG individually or concomitantly and performed a preliminary co-immunoprecipitation experiment. As a validation of the Y2H data, we found that immunoprecipitation of mSAN1-FLAG was indeed able to interact with MYC-mPIAS1 (Figure 23a).

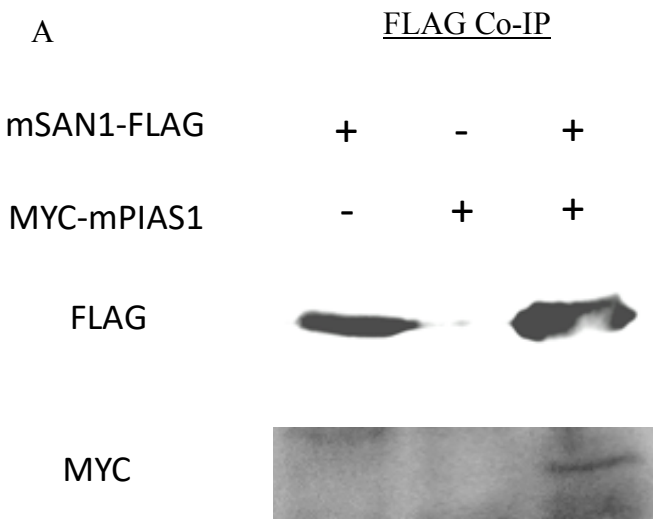

Figure 23. SAN1 interacts with the E3 SUMO ligase PIAS1. (a) Western blot showing co-immunoprecipitation experiments of mouse SAN1-FLAG and a MYC-tagged mouse PIAS1 construct that were over-expressed in HEK 293T cells.

Further experiments are needed to validate this preliminary data, but it opens up some intriguing lines of further investigation. In the future it would be informative to investigate if SAN1 is indeed sumoylated by PIAS1, the site of sumoylation on SAN1 that this post translational modification might occur, and if this PTM or interaction with PIAS1 is necessary for SAN1 function in ICL repair.

Impact of cell cycle stage on SAN1

Another possibility worth investigating involving the potential regulation of SAN1, is the question of if SAN1 activity or expression is cell cycle specific. Cell cycle regulation is critical for numerous DNA repair proteins, as well as for a cell's ability to influence overall DNA repair pathway choice within the DNA damage response (Reinhardt and Yaffe et al., 2013). To explore this possibility we synchronized HeLa cells using the inhibitor L-Mimosine which arrests cells in late G1 at the G1/S phase boundary (Krude et al., 1999). We then washed the cells of L-Mimosine and allowed them to progress to late S phase (Maity et al., 1995). Strikingly, analysis by western blot demonstrated that SAN1 protein levels were much higher in late S phase, compared to G1 or in asynchronous cells (Figure 24a).

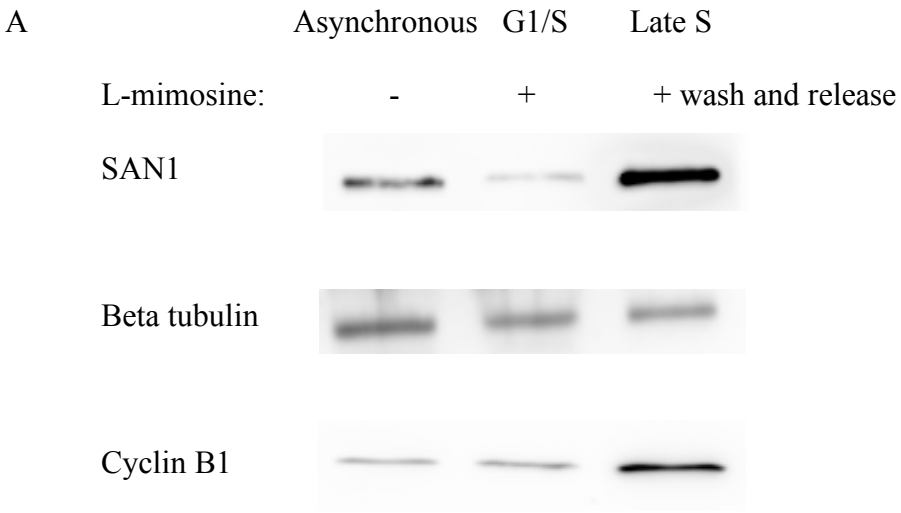

Figure 24. Effect of cell cycle stage on SAN1 expression. (a) Western blot showing expression levels of SAN1 at different time points in the cell cycle. HeLa WT cells were arrested in G1 by treatment with L-mimosine, and either lysed or washed and released for 9 hours to progress to late S phase. Cyclin B1 was used to assess cell cycle stage and Beta tubulin was used as a loading control.

Although this is preliminary data the result is very interesting as it opens up the potential for multiple new lines of investigation. The change in protein levels of SAN1 will need to be validated by more replicate experiments, as well as alternative independent methods of inducing cell cycle arrest to ensure this phenotype is not an off target effect of L-Mimosine treatment. In future studies it would be interesting to determine the mechanism by which SAN1 protein levels are increased and restricted to late S phase, as well as examining the expression level of SAN1 in G2 and M phases of the cell cycle. SAN1 expression might be induced and upregulated as cells progress from G1 to S phase, or SAN1 might be expressed at levels similary throughout the cell cycle and kept low in G1 through K63- polyubiquitylation and degradation (Wilkinson et al., 2000). It also raises the question of if the activity of SAN1 varies in different cell cycle stages.

Additionally the high expression level of SAN1 in late S phase might hint at the underlying mechanism through which SAN1 participates in ICL repair. It has been speculated and some evidence suggests, that transcription dependent ICL repair mechanisms operate primarily in G1 of the cell cycle, whereas replication dependent repair pathways such as the FA pathway dominate the response to ICLs in S and G2 phases of the cell cycle (Williams, Gottesman, and Gautier et al., 2012). Our current data concerning whether SAN1 participates in transcription or replication dependent ICL repair is inconclusive. However, if these initial results are correct that SAN1 expression is high in S phase, that might suggest a possible involvement of SAN1 in some type of replication dependent ICL repair process.

Co-localization of SAN1 with DDR markers

A key feature of many proteins within the DNA damage response is their localization to discrete foci following the induction of DNA damage. For many DNA repair proteins this typically occurs as a transition from a diffuse localization to discrete foci that mark the sites of DNA lesions (Bekker-Jensen et al., 2006). FANCD2, a core component of the FA pathway, re-localizes from a diffuse staining pattern to discrete foci that are believed to mark the sites of ICLs (Garcia-Higuera et al., 2001). In order to examine the localization of SAN1, we attempted staining of endogenous SAN1 using several antibodies and various fixation and staining conditions, but due to the low expression of SAN1 we were unable to confidently assess SAN1 localization above background levels in these experiments. Therefore we over-expressed SAN1-FLAG in HeLa cells to assess the localization of SAN1 before and after DNA damage, as well as to determine if we could viusalize SAN1 potentially localizing to ICL sites (Figure 25a).

A

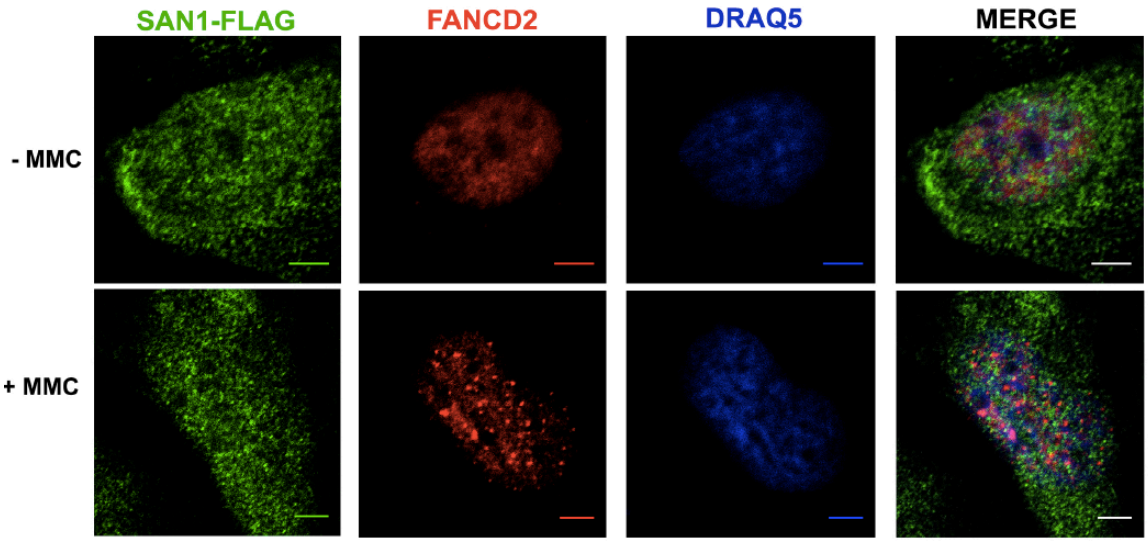

Figure 25. Localization of SAN1-FLAG following DNA damage. (a) IF images HeLa WT cells over-expressing a SAN1-strep^2-FLAG construct before and after DNA damage.

Cells were treated with vehicle or MMC for 24 hours and stained for FLAG, FANCD2, and DRAQ5.

In contrast to the re-localization from a diffuse staining pattern to discrete foci like FANCD2, SAN1-FLAG displayed a mixture of diffuse staining and small foci that are constitutively present (Figure 25a). Surprisingly, there was no clear change in the localization of SAN1-FLAG after treatment with MMC. In some instances, SAN1-FLAG and FANCD2 foci appear to co-localize, however this might be merely a result of constitutive SAN1-FLAG foci and inevitable overlap because of the high number of foci, rather than an indication that SAN1-FLAG is actually localized to the site of an ICL.

These data are somewhat difficult to interpret, as the over-expression of SAN1 in these experiments may be the cause of the constitutive foci formation that we observe. Similar results were obtained examining the localization of SAN1-FLAG and 53BP1 or γH2AX after treatment with MMC (Figure 26 and 27), two markers of DSBs (Rappold et al., 2001) (Burma et al., 2001).

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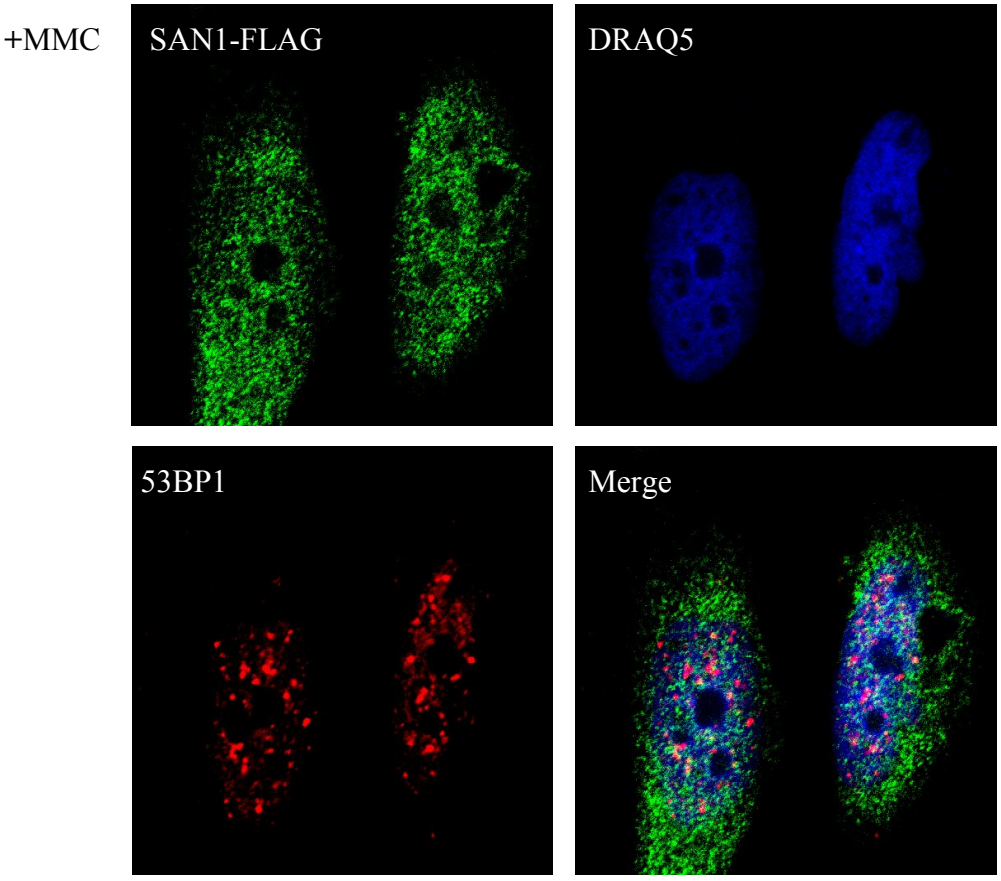

Figure 26. Potential co-localization of SAN1-FLAG and 53BP1 foci. (a) Immunofluorescence images HeLa WT cells over-expressing a SAN1-strep^2-FLAG construct after treatment with MMC. Cells were treated MMC for 24 hours and stained for FLAG, 53BP1, and DRAQ5.

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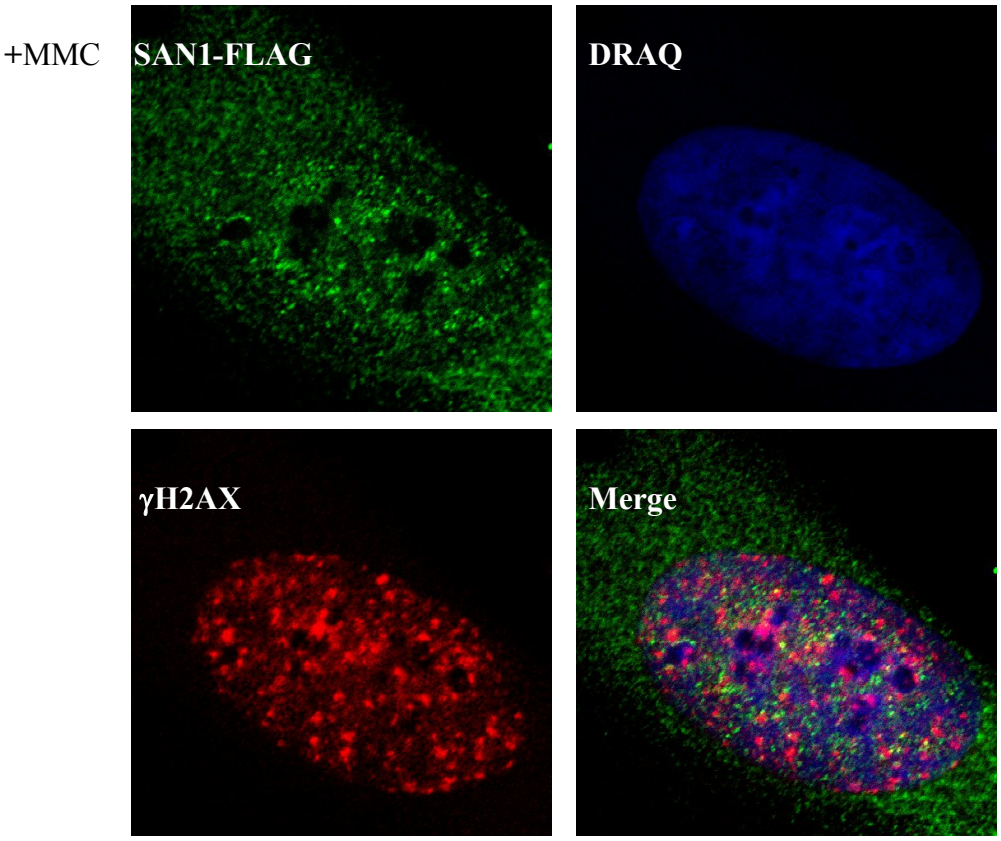

Figure 27. Possible co-localization of SAN1-FLAG and γH2AX foci. (a) IF images HeLa WT cells over-expressing a SAN1-strep^2-FLAG construct after treatment with MMC. Cells were treated MMC for 24 hours and stained for FLAG, γH2AX, and DRAQ5.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

The studies described in this dissertation provide novel findings concerning the mechanisms by which interstrand cross-links are repaired. In this work have identified and characterized a novel nuclease of the FEN1 superfamily of structure specific nucleases. Fam120b, a previously uncharacterized gene that has previously only been described as a transcriptional co-activator involved in adipogenesis, is in fact a 5' ssDNA exonuclease that functions in the cellular response to ICLs. A previous characterization of Fam120b implicated this gene in the process of transcriptional regulation, and suggested the name of constitutive co-activator of peroxisome proliferator-activated receptor gamma (CCPG) (Li et al., 2001). However, RNA-seq studies performed by us in Fam120b +/+ and -/- mouse embryonic fibroblasts demonstrated no significant changes in gene expression (data not shown). Instead we found that the Fam120b gene product displays specific 5' exonuclease activity towards ssDNA and splayed arm substrates. Moreover, Fam120b interacts with the RNA/DNA helicase Senataxin, an interaction that we demonstrate is required for Fam120b function in ICL repair. For these reasons we have proposed the name SAN1, for Senataxin associated nuclease 1.

Following the discovery of SAN1, we demonstrated that SAN1 does in fact display nuclease activity that is specific to the purified protein, and can be ablated by mutation of a single conserved residue within the catalytic site of the conserved FEN1 family nuclease domain (D90). This information led us to investigate the type of DNA damage and repair that SAN1 might function in and be required for. Utilizing a CRISPR/Cas9 generated knockout HeLa cell line, as well as SAN1-/- MEFs, we found

that the loss of SAN1 sensitized cells specifically to ICLs but not other forms of DNA damage. Moreover, the nuclease activity of SAN1 was essential to restore resistance to the cross-linking agents, indicating that the nuclease function is necessary for its role in the cellular response to ICLs.

To investigate if the sensitivity of SAN1 deficient cells to cross-linking agents was a direct result of damaged DNA, we collaborated with the D'Andrea lab to have cytogenetic analyses performed. Strikingly, we found that SAN1-/- cells treated with MMC displayed very high levels of radial chromosomes and chromosomal aberrations, a hallmark of Fanconi anemia and cell defective in ICL repair (Moldovan and D'Andrea et al., 2009). In fact the presence of radial chromosomes is a type of DNA damage that is specifically caused by unrepaired ICLs, and is direct evidence of the involvment of SAN1 in the response to cross-linking agents. In agreement with this data we also observed that SAN1-/- cells displayed elevated levels of γH2AX and 53BP1 foci, markers of DNA damage and double strand breaks (Rappold et al., 2001) (Burma et al., 2001).

Given these data support an involvement of SAN1 in ICL repair, we investigated the relationship of SAN1 to the canonical repair pathway for ICLs, the Fanconi anemia pathway. Strikingly, despite the Fanconi-like phenotype of radial chromosomes in response to MMC, we found that SAN1 is not epistatic to FANCD2 or XPF, indicating that SAN1 functions independently of the FA pathway. Interestingly however, we found that SAN1 is in fact epistatic to the 5' nucleases SNM1a and FAN1, suggesting it might cooperate with these nucleases to repair ICLs in a FA independent mechanism. We also demonstrated that the SAN1 loss of SAN1 does not afffect FANCD2 foci formation or

mono-ubiquitylation, ruling out the possibility that the sensitivity of SAN1-/- cells to ICLs might be a result of an indirect effect on the FA pathway.

As our data suggested an involvement of SAN1 independently of the FA pathway in ICL repair, we sought to identify interacting proteins that might inform us about the mechanims through which SAN1 functions. A yeast two hybrid screen identified the RNA/DNA helicase Senataxin as a potential binding partner, an interaction that we validated by endogenous co-immunoprecipitation experiments. Strikingly, we found that this interaction between SAN1 and Senataxin was enhanced in the presence of ICLs, and that SAN1 binding to Senataxin was essential for preventing sensitivity to cross-linking agents.

The finding that SAN1 and Senataxin cooperate in a repair pathway that responds to ICLs, led us to investigate the potential relationship of R-loops to this process. R-loops, are RNA/DNA hybrids that Senataxin acts to unwind as one of its central functions (Skourti-Stathaki et al., 2011). Surprisingly we found that R-loop levels are increased in SAN1-/- cells, particularly following treatment with MMC.

A central question that remains to be definitively answered in our work, is whether SAN1 plays a direct role in the processing of R-loops, or if increased R-loop levels are a secondary consequence of the inability of SAN1-/- to repair some ICLs. This in turn would likely cause increased transcriptional stalling and R-loop formation. We believe our data supports a model in which SAN1 participates in the repair of ICLs, rather than in R-loop resolution. Notably, the presence of radial chromosomes indicates direct evidence of unrepaired ICLs, as these structures result from the formation of one-sided DSBs that are aberrantly joined to other chromosomes in a NHEJ dependent

process (Deans and West et al., 2011). Along these lines, while persistent R-loops have been shown to cause DNA damage and increase genomic instability, they have never been shown to lead to radial chromosomes. An additional alternative possibility, is that the binding of SAN1 to Senataxin influences the helicase activity of SETX towards R-loops, thereby increases there presence in untreated and MMC treated conditions when SAN1 is lost. Future studies, including if SAN1 displays any *in vitro* biochemical activity towards RNA/DNA hybrids are needed and would be highly informative.

Collectively, our data support a speculative model where Senataxin might serve as a means of recruitment to ICL sites. In this model, R-loops that form adjacent to an ICL from transcriptional stalling, might recruit Senataxin in order to be resolved, thus providing a mechanism by which SAN1 is also recruited through its interaction with SETX. Following the generation of a single strand break, SAN1 might then act on the displaced ssDNA loop structure, or after re-annealing of the ssDNA on a subsequent 5' splayed arm structure. This nuclease activity of SAN1 might provide a better substrate for SNM1a and FAN1 to the digest past the ICL site, unhooking it from the 5' side. It is tempting to speculate that stalling of a transcription complex near an ICL might present a scenario in which an alternative ICL repair mechanism than the FA pathway would be required. It has been shown that the FA pathway at least in the biochemical Xenopus system, requires the dual convergence of replication forks for activation, a process that the presence of a stalled RNA pol II complex and R-loop would prevent (Zhang et al., 2015). This type of transcription replication conflict near an ICL might require the functions of Senataxin and then SAN1 to fully respond to ICLs.

While our work demonstrated here greatly furthers our understanding of the mechanisms and pathways by which cells respond and repair ICLs, there are numerous areas that warrant further investigation. One question of critical importance, but difficult to directly address, is aiming to determine the precise step within ICL repair that SAN1 acts. This area of investigation might be best explored through an *in vitro* approach, and may require the partial biochemical reconstitution of the ICL repair process that SAN1 particpates in. These experiments could theoretically be conducted in the Xenopus extract system, but the role of SAN1 in this system remains undefined and initial depletion experiments should be carried out first. Of particular interest and relevance to these questions is whether or not SAN1 can directly digest past or unhook a cross-linked substrate, a question despite preliminary biochemical experiments still remains unclear (data not shown).

Also directly relevant to defining the step that SAN1 plays in ICL repair, is addressing whether SAN1 participates in a replication or transcription dependent ICL repair process. Although we clearly established that SAN1 cooperates with Senataxin in ICL repair, a protein that plays a central role in transcription coupled repair processes like R-loop resolution, it remains unclear if the role of SAN1 in ICL repair is indeed dependent on transcription. However it seems unlikely that SAN1 instead functions in a replication dependent process, given the non-epistatic relationship to the FA pathway, and that SAN1-/- proliferate normally and do not display cell cycle abnormalities (data not shown).

An additional line of exploration that we have not addressed, is the potential regulation of SAN1 in ICL repair. As discussed previously, cell cycle control,

ubquitylation or sumolylation, and phosphorylation all are reasonable means by which the activity of SAN1 in ICL repair might be regulated. These potential PTMs might also affect localization of SAN1 or association with Senataxin. Given that nearly all DDR pathways are tightly regulated to ensure genome stability, this will likely be an important area of future research.

In summary the work described here provides compelling insights into mechanisms of ICL repair, through the characterization of a novel nuclease SAN1. Our data that we have presented improve the overall understanding of how cells respond to interstrand cross-links, and the proteins and mechanisms by which they do so. Further research in these areas will be important to expand our knowledge of DNA repair mechanisms and how they contribute to genome stability and prevent diseases including cancer.

METHODS

Plasmids and siRNA. Human SAN1 cDNA (FLJ56631) was purchased from the NBRC, Japan, and cloned into the pRK7 expression vector with a C-terminal FLAG tag. SAN1 (D90A) and SAN1 rescue constructs were made by QuikChange site-directed mutagenesis (Agilent Technologies). Partial mouse SAN1 open reading frames were obtained from GE Healthcare Dharmacon MGC cDNAs library and cloned into pASK-IBA3Plus to express full length SAN1-Strep-tag II. The D90A mutation was introduced using Stratagene QuikChange. pICE-RNaseHI-WT-NLS-mCherry was a gift from Patrick Calsou (Addgene plasmid # 60365). For RNAi knockdown experiments SMARTpool siGENOME (Thermo Fisher) siRNAs were used for FANCD2, SETX, SNM1A, XPF, FAN1, and FAM120B. Cells were transfected, split 24 hrs later, and transfected again, with specific siRNA pools or a scrambled control using RNAiMAX (Invitrogen) and Opti-MEM media (Thermo Fisher) according to manufacturer's instructions. Colony survival assays were performed and lysates made 36 hrs after the second round of siRNA transfection. XPF (Bethyl A301-315A) and SNM1a (Bethyl A303-747A-M) antibodies were used at 1:1000 for western blotting.

Cell Culture and Transfections. HeLa (ATCC) and 293T (ATCC) cells were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin (GIBCO). Transfection of plasmids or siRNA was performed with calcium phosphate, Lipofectamine 2000 (Invitrogen), or Lipofectamine RNAiMAX (Invitrogen). Virus was

made, collected and titered as described previously (McCaffrey et al. 2009). Cell lines verified mycoplasm free by DAPI staining. Cell lines validated by DNA sequencing.

Colony survival assays. HeLa or mouse embryonic fibroblast cells were seeded at 300-400 cells per 6 well dish for ~16 hrs overnight, treated with DNA damaging agents, and allowed to form colonies for 7-10 days. Colonies were fixed in ice cold 70% EtOH, stained with crystal violet, and counted.

Production of SAN1$^{-/-}$ HeLa cells. Two 60-bp guide sequences (sgRNA1 F: 5′-TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCAGGATAAGAG AGATGAAT -3′ and sgRNA2 F: 5' – TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGAGAAGCTCTGTG AGAGTCT – 3') were designed to target sites in the first exon of SAN1, to remove the majority of the nuclease domain. The sgRNA1 and sgRNA2 were cloned into gRNA cloning vectors and verified by sequencing. gRNA1, gRNA2, and NLS-hCas9-NLS constructs were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen 11668-030) per Invitrogen protocol publication number MAN0007824 Rev 1.0 and seeded at single cell density on 15 cm dishes. Individual colonies were isolated by 0.25% trypsin and were plated separately in 6 well plates to grow. Genomic DNA was isolated from WT HeLa cells and from 12 clones. Exon 1 of SAN1 was PCR amplified using SAN1 genomic PCR primers (SAN1 genomic F: 5' – ACTGATTAATTTATCTTTCTTTCCAGATCC – 3' and SAN1 genomic R: 5'TCTGGGATTATGTCGTTGCCAAGGAGG – 3') and clones that showed a deletion

were sequenced and analyzed by immunoblot (SAN1 1:1000). 6 SAN1$^{-/-}$ HeLa clones were identified and experiments in this study were completed with SAN1$^{-/-}$ HeLa Clone 2 unless otherwise specified.

Generation of immortalized Mouse Embryonic Fibroblasts (MEFs). A conditional KO mouse was created through the Texas A&M Institute for Genomic Medicine, using EUCOMM ES cells targeting the *fam120b* gene (ES cell clone HEPD0652_5_G10). A *fam120b*/+ male was crossed to a FLPer/+ female to delete the lacz/neo markers, the FLPer transgene was then removed by crossing to a +/+ mouse, and the resulting floxed allele mice were crossed to produce homozygotes. These were then crossed with a Sox2-Cre mouse to obtain a global knockout of the allele. *Fam120b* +/+ and -/- MEFs were isolated from day 13.5 embryos, from matings of heterozygous parents. Embryos were incubated in 0.25% Trypsin overnight at 4° C for digestion to single cells. Cells were plated and cultured in 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin (GIBCO). MEFs were immortalized by transfection with a plasmid encoding SV40 Large T antigen (Addgene 21826), followed by a 1/10 split of the cells 5-6 times.

Immunofluorescence staining and Microscopy. FANCD2 staining: Cells were seeded in 8 well chamber Labtek II slides and treated with vehicle or 120 ng/mL MMC for 24 hrs. Cells were washed twice with 1X PBS, and fixed in 4% paraformaldehyde for 15 min. Cells were then blocked in 3% BSA with 0.3% Triton TX-100 for 1 hr, then incubated with rabbit anti-FANCD2 (1:500, Novus Biologicals NB100-182SS) antibody for 1 hr at room temp. Cells were washed 3X for 5 min with 1X PBS then incubated with

594 Goat anti rabbit (1:500, Alexa Fluor) secondary antibody, and DRAQ5 nuclear stain (cell signaling tech. 4084S) for 1 hr at room temp. Slides were mounted in Fluoromount G and sealed with nail varnish. Confocal images were acquired using a Nikon A1R confocal microscope using a 60x oil lens (na 1.3). For R-loop (S9.6) and Nucleolin staining, HeLa WT and SAN1-/- cells were treated with vehicle or 1 μM MMC for 30 hrs, and fixed in 4% paraformaldehyde, blocked in 3% BSA for 1 hr at room temp, and incubated with mouse monoclonal S9.6 (Kerafast, 1:200 ENH001) and rabbit polyclonal anti Nucleolin (Abcam 1:1000 ab22758) antibodies overnight at 4° C. Cells were washed 3X for 5 min with 1X PBS and then incubated with 488 Goat anti mouse (1:500, Alexa Fluor) and 594 Goat anti rabbit (1:500, Alexa Fluor) secondary antibodies, and DRAQ5 nuclear stain (Cell Signaling Tech. 4084S) for 1 hr at room temp. Slides were then mounted in Fluoromont G and sealed. For γH2AX and 53BP1 staining cells were washed twice with 1X PBS, fixed in 4% paraformaldehyde for 15 min, blocked in 3% BSA with 0.3% Triton TX-100 for 1 hr at room temp, and incubated with γH2AX (Ser139 Millipore 1:500, 05-636) or 53BP1 (Bethyl 1:500, A300-272A) for 1 hr at room temperature. Cells were washed 3X for 5 min with 1X PBS and then incubated with 488 Goat anti mouse or 594 Goat anti rabbit (1:500, Alexa Fluor) secondary antibodies, and DRAQ5 nuclear stain (Cell Signaling Tech. 4084S) For the RNaseH1-mCHerry experiment, cells were transfected with the pICE-RNaseHI-WT-NLS-mCherry construct using lipofectamine 2000, and incubated with 10 μg/mL of doxycycline for 24 hours to induce expression. Cells were then treated with vehicle 1 μM MMC for 30 hrs and stained for γH2AX and DRAQ5 as described above.

Radial chromosome assays. HeLa cells were incubated for 48 hrs in the presence or absence of MMC. Colcemid (0.1 g/ml) was added to the medium 2 hrs before the cells were collected. For each sample, 50 metaphases were analyzed for chromosomal abnormalities, as previously described (Shimamura et al., 2002). Data were analyzed in Prism GraphPad from contingency tables using Fisher's exact-test (two-sided P value)

Co-immunoprecipitation of SAN1 and Senataxin. HeLa cells were fractionated as previously described (Huang et al. 2009), with the following modifications. Cells were washed once with 1X PBS and the pellet was resuspended in 2-5 volumes of lysis buffer (10 mM HEPES pH 7.4, 10 mM KCl, 0.05% NP-40) containing Roche protease and phosphatase inhibitors, and incubated on ice for 20 min. Lysates were centrifuged at 13000 rpm at 4° C for 10 min. The supernatant containing the cytoplasmic proteins was discarded, and the pellet containing the nuclei was washed once with lysis buffer and resuspended in Low Salt Buffer (10 mM Tris-HCl pH 7.4, 0.2 mM $MgCl_2$), incubated on ice for 15 min, and centrifuged for 10 min at 4° C. The supernatant was removed and combined with an equal volume of 2X Co-IP buffer (20 mM HEPES pH 7.4, 200 mM NaCL, 1% Triton Tx-100, 1 mM EDTA, 10 mM $MgCl_2$) and placed on ice. The soluble fraction was incubated for 1.5 hrs rotating at 4° C with Strep-Tactin agarose beads (IBA) to immunoprecipitate (IP) over-expressed SAN1ssf, or with rabbit polyclonal anti-FAM120b antibody (abcam 1:1000, ab106455) and protein A/G Sepharose to precipitate endogenous SAN1. Samples were washed 3X for 5 min in 1X Co-IP buffer, and eluted with 10 mM desthiobiotin for 2 hrs at 4° C (SAN1-ssf), or by boiling samples in 4X Laemmli sample buffer. Co-IP samples and inputs were analyzed by immunoblot with

SAN1(1:1000, abcam ab106455), SETX (1:1000, Novus NB100-57542), FLAG (1:1000, Sigma F1804), GFP (1:500 Abcam Ab13970), and Phospho-Chk2 (1:500, Cell signaling tech 2661) antibodies. 3% milk was used for blocking.

Co-immunoprecipitation of SAN1-FLAG and myc-SETX-SID fragment. 293T cells were co-transfected using calcium phosphate with either SAN1-FLAG, SAN1ΔRep-FLAG, and myc-SETX-SID **fragment,** and lysed in buffer containing 25 mM HEPES pH 7.4, 150 mM NaCl, 0.5% Triton-X 100, 0.5 mM EDTA, 1 mM $MgCl_2$, 2 mM DTT, 1 mM PMSF, 10 ug/ml leupeptin, 20 ug/ml aprotinin 24 hrs later. Cell lysates were then incubated with anti-FLAG M2 agarose for 1 hr at 4°C rotating before 3 washes. Samples were boiled for 8 min in 4X LSB and analyzed by immunoblot for FLAG (M2 Sigma F1804) and (Myc 9E10) antibodies at 1:1000.

Cell cycle analysis. HeLa cells were transfected with indicated siRNA and then treated with vehicle or 5 μM Cisplatin for 2 hrs, replaced with fresh media and 24 hrs later harvested for flow cytometric analysis. Cells were washed in PBS and then fixed in ice-cold 70% ethanol for at least 2 hrs. The cells were washed in PBS and resuspended in propidium iodide staining solution (PBS, 0.1% TritonX-100, 0.2 mg/mL DNase-free RNase A (Sigma), 20 μg/m LPI (Sigma)) and analyzed using a FACSCalibur machine (BD).

Yeast-two hybrid screen. The yeast two-hybrid screen using full-length murine SAN1 as bait was carried out by Hybrigenics Corporation, Cambridge, MA using a mouse brain

library (Supplementary Figure 5A related to Figure 7).

RNA/DNA hybrid slot-blot assay. Hybrids were detected as described by Sollier et al (2014). Total nucleic acids were extracted from cells with the Qiagen DNeasy kit. DNA (1 ug) was spotted in duplicate wells onto positively-charged nylon membrane using a slot-blot apparatus, cross linked by UV treatment, and one well was probed with the S9.6 antibody (1:1000, Kerafast, 1:200 ENH001). The DNA in the duplicate well was denatured for 10 min in 0.5N NaOH, 1.5M NaCl, then neutralized for 10 min in 1M NaCl, 0.5 M Tris-HCl (pH 7.0) and probed with an antibody against ssDNA (Millipore MAB3034; 1:10000). Spots were detected with an anti-HRP secondary anti-mouse antibody, and imaged and quantified with an Amersham Imager 600.

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