ELUCIDATING THE MECHANISMS SURROUNDING H2A.X MONOUBIQUITYLATION MARKS IN THE DNA DAMAGE RESPONSE PATHWAY

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ABSTRACT

In response to DNA double-strand breaks (DSBs), H2A.X undergoes three post-translational modifications (PTMs) reported to be important for DSB signaling: phosphorylation at serine 139, monoubiquitylation at lysines 118/119 and monoubiquitylation at lysines 13/15. We aimed to gain a better understanding of the mechanisms surrounding H2A.X monoubiquitylation marks and their relationship to H2A.X phosphorylation. We have developed and tested a novel purification method for the isolation of ubiquitylated H2A.X-containing nucleosomes, which can be used to co-purify DNA damage-response proteins that interact with ubiquitylated H2A.X-containing nucleosomes. Our data show that monoubiquitylation of lysines 118/119 is not required for phosphorylation at serine 139, nor for monoubiquitylation at lysines 13/15 *in cis* (on the same H2A.X molecule). Our data also suggests that monoubiquitylation of lysines 118/119 may have an antagonistic effect on monoubiquitylation of lysines 13/15. Lastly, we saw that different DSB-inducing agents can have different effects on H2A.X monoubiquitylation.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

γ-H2A.X: H2A.X phosphorylation at serine 139

53BP1: p53-binding protein 1

alt-EJ: alternative end-joining

ATM: Ataxia Telangiectasia Mutated

ATR: Ataxia Telangiectasia and Rad3 Related

AVI-HRP: Avidin – horseradish peroxidase

BICON: Biotinylation-assisted Isolation of Co-modified Nucleosomes

bp: base pair

BRCA1: Breast Cancer Type I Susceptibility protein

BSA: bovine serum albumin

CDK: cyclin-dependent kinase

CtIP: CTBP interacting protein

DMSO: dimethyl sulfoxide

DNA-PK: DNA-dependent protein kinase

Dox: doxorubicin

DSB: double-strand break

***E. coli*:** *Escherichia coli*

ECL: enhanced chemiluminescence

H2A.X$^{-/-}$ MEFs: H2A.X null mouse embryonic fibroblasts

HR: homologous recombination

IP: immunoprecipitation

IR: ionizing radiation

kDa: kilodalton

MDC1: Mediator of DNA Damage Checkpoint protein 1

MMS: methyl methanesulfonate

MNase: micrococcal nuclease

MRN: MRE11-RAD50-NBS1

MSK1: Mitogen and Stress-Activated Protein Kinase 1

n: sample size

NCS: neocarzinostatin

NHEJ: non-homologous end joining

PARP1: Poly(ADP-Ribose) Polymerase I

PBS: Phosphate Buffered Saline

PCR: polymerase chain reaction

PCR1: Polycomb Repressive Complex 1

PEI: Polyethylenimine

PP4, PP6: protein phosphatase 4, 6

PTIP: Pax Interacting Protein 1

PTM: post-translational modification

PVDF: Polyvinylidene Fluoride

RAP80: Receptor-Associated Protein 80

Rb: Retinoblastoma

Rif1: Replication Timing Regulatory Factor 1

RNF168, RNF8: Ring Finger Protein 168, 8

RNF2/RING1B: Ring Finger Protein 2

RT: room temperature

SDS: sodium dodecyl sulfate

sgRNA: subgenomic RNA

SSA: single-strand annealing

SSB: single-strand break

SV40: Simian Virus 40

TBS: Tris Buffered Saline

TBS-T: Tris Buffered Saline with Tween

TE: Tris-EDTA

Ub: ubiquitin/ubiquitylated

USP3, USP16, USP51: Ubiquitin Specific Peptidase 3, 16, 51

Wip1: wild-type p53-induced phosphatase 1

WT: wild-type

CHAPTER 1: INTRODUCTION

Our DNA contains all the information required for proper cell functioning; genes are transcribed to messenger RNA molecules, which eventually become translated to generate proteins with specific purposes (Dynan and Tjian, 1985). Therefore, it is essential that our DNA remains in an intact and healthy state to ensure proper functioning as a whole organism. However, DNA is susceptible to various endogenous and exogenous sources of damage (Helleday et al., 2014) with the most dangerous type being DNA double-strand breaks (DSBs). DNA DSBs are harmful to cells because they can cause a series of complications that often leads to the development of cancers (Pan et al., 2011). Fortunately, when a DNA DSB occurs, cells have ways of sensing the location of the break, which leads to a signaling cascade that recruits proteins that are able to repair the lesion. Histone H2A.X is a DNA-associated protein that plays a significant signaling role leading to the recruitment of DNA repair proteins. It has been well known for many years that H2A.X becomes phosphorylated at a specific serine residue (S139 in human H2A.X) to help signal the recruitment of repair proteins (Lin et al., 2015). In more recent years, it has been discovered that H2A.X also becomes monoubiquitylated (modified by a single ubiquitin moiety) at two distinct sites, and these two monoubiquitylation modifications have also been reported to play distinct signaling roles in the recruitment of DNA repair proteins (Pan et al., 2011; Mattiroli et al., 2012). However, unlike phosphorylation of H2A.X, the mechanisms surrounding monoubiquitylation modifications on H2A.X are less well understood, and are therefore of interest to the field. This introduction will provide an overview of where H2A.X resides in the cell, what is currently known about H2A.X phosphorylation and monoubiquitylation, and the specific objectives of this project.

1.1 Chromatin Structure

The genetic material in eukaryotic cells is organized as a complex of DNA and proteins, collectively referred to as chromatin (Sajan and Hawkins, 2012). The main constituents of chromatin are the nucleosomes, which are individual DNA-protein complexes that play a role in packaging DNA into the nucleus of a eukaryotic cell **(Figure 1)**. The core of each nucleosome is composed of an octamer containing four types of core histones: H3, H4, H2A and H2B. Formation of a typical nucleosome starts with two H3-H4 dimers joining to form a tetramer, and two H2A-H2B dimers added to either side of the tetramer. Approximately 147 base pairs (bp) of DNA is then wrapped around each histone octamer to complete the nucleosome (Suganuma and Workman, 2011). The distance between any two nucleosomes varies between 20-80 bp, depending on the length of linker sequence that connects one core to the other (Fuchs and Oren, 2014).

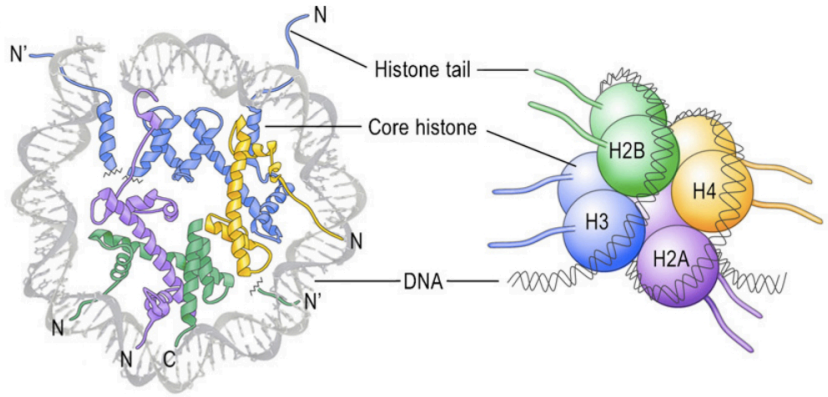


FIGURE 1. The structure of a nucleosome. Each nucleosome is composed of an octamer containing four types of histones. Each nucleosome contains two copies of histones H2B, H2A, H3 and H4. These histones contain "tails", which are amino acid chains that extend outward from the globular domain of histones. Wrapped around each octamer is ~147 bp of DNA. Figure adapted from Gräff and Mansuy 2008.

In its simplest form, chromatin is arranged as an 11 nm fiber. The 11 nm fiber adopts a conformation similar to that of "beads on a string" as it is commonly referred to, where nucleosomes are positioned in a linear fashion along a stretch of DNA. However, the 11 nm fiber can fold up into a more compact, coiled 30 nm fiber with the assistance of histone H1. Unlike the four histones that comprise the core of the nucleosome, H1 binds the linker DNA in between nucleosomes to help condense the 11 nm fiber into the 30 nm form. The 30 nm fiber itself can condense into a variety of higher-order chromatin structures that culminates to the most compacted state: the metaphase chromosome (compacted 10,000 fold) **(Figure 2)**. The physical state of chromatin within the nucleus of a cell can vary throughout different parts of the genome, and at different time points in the cell cycle. The level of compaction of a region of DNA correlates with the type of information it encodes. Open, uncondensed regions (euchromatin) usually contain genes that are actively transcribed, which reflects the fact that transcription factors need to access the underlying DNA in order to drive transcription. For that same reason, closed and condensed regions (heterochromatin) are generally transcriptionally inactive. Examples of such regions include the centromeres and telomeres of individual chromosomes (Sajan and Hawkins, 2012).

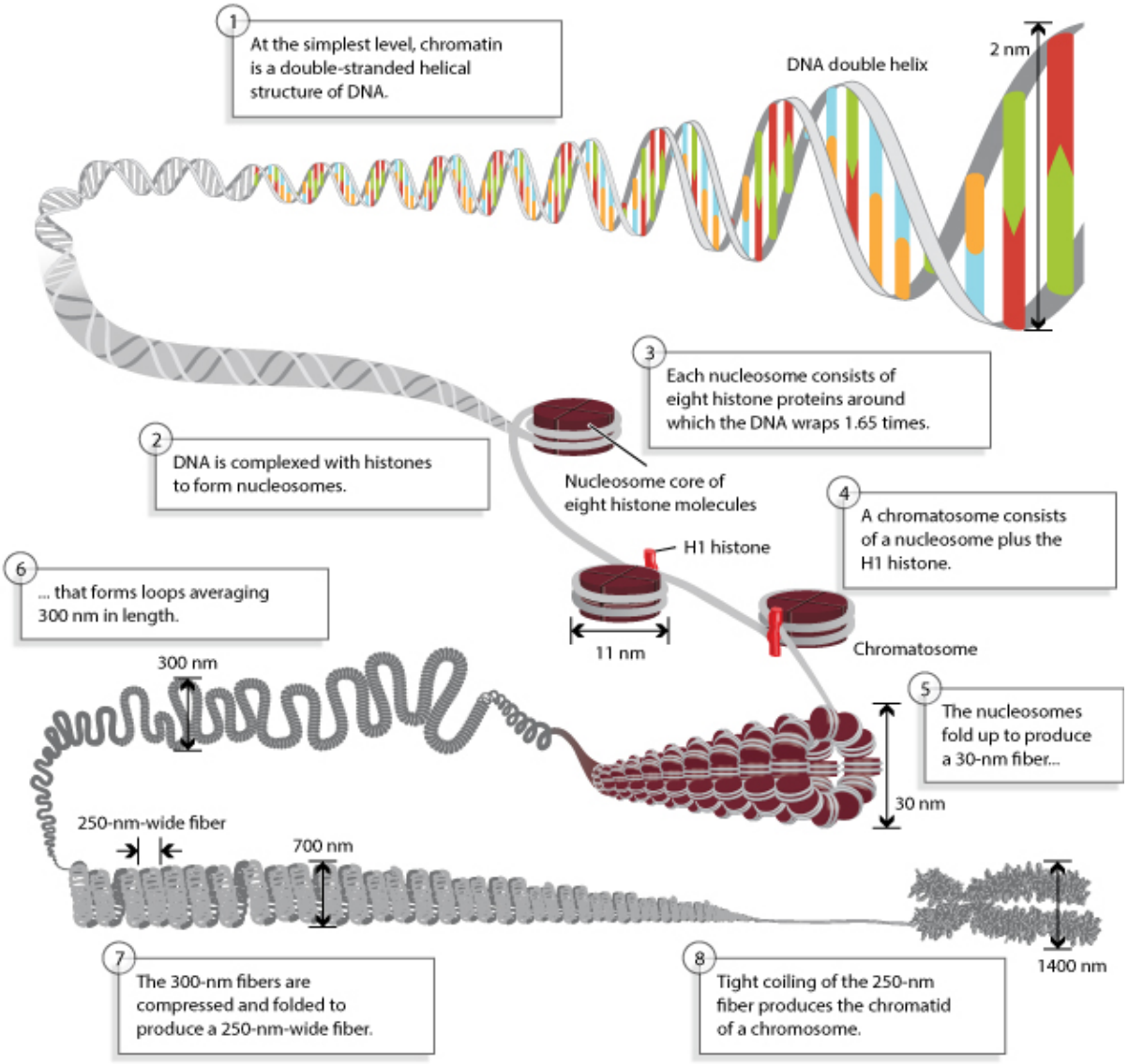


FIGURE 2. Different levels of chromatin compaction. DNA is wrapped around an octamer of histones to form a nucleosome. In its simplest form, nucleosomes are arranged in a linear fashion, referred to as an 11 nm fiber. Histone H1can bind to the linker sequences between nucleosomes to help compact the 11 nm fiber into a 30 nm fiber. The 30 nm fiber can further be compacted to generate more condensed chromatin structures that lead to the eventual formation of a chromatid. Figure adapted from Pierce, Benjamin. *Genetics: A Conceptual Approach*, 2nd ed.

1.2 Epigenetic Modifications

The physical state of chromatin is regulated by epigenetic modifications. Epigenetics is the study of heritable changes that are not mediated at the DNA sequence level. The epigenetic perspective differs from that of classical genetics, which narrowly defines that the information potential of the genome is only dependent on the DNA sequence. Epigenetic modifications can regulate the physical state of chromatin, as well as downstream signaling events in the cell. Epigenetic modifications to chromatin occur in two forms: DNA methylation and histone post-translational modifications (PTMs) (Bernstein et al., 2007).

1.2.1 Histone PTMs

There are many different PTMs that can occur on histones, including methylation, phosphorylation, and ubiquitylation (the addition of a methyl group, phosphate group, and ubiquitin molecule, respectively). Histone PTMs usually occur on the histone N-terminal and C-terminal "tails", which consist of amino acid chains that extend outward from the globular domain of histones (Bernstein et al., 2007) **(Figure 3)**. Modifications on histone tails can affect chromatin structure in multiple ways. For example, histone PTMs can cause nucleosomes to be repositioned in chromatin by recruiting modeling enzymes (Swygert and Peterson 2014). The charge-based interactions between histones and DNA can also be disrupted by histone PTMs. Although histone modifications regulate cellular processes largely by altering the physical structure of chromatin, they can also act to recruit specific proteins ("readers") that cause downstream signaling events (Fuchs and Oren, 2014). Crosstalk among different PTMs on histones can also occur, where one PTM can influence the presence of another. Crosstalk occurs in two forms: cis-crosstalk (between modifications on the same histone) and trans-crosstalk

(between modifications on different histones) (Zhang et al., 2014) **(Figure 3)**. Collectively, the idea that combinations of histone PTMs help regulate the information potential of the genome is referred to as the "histone code hypothesis" (Suganuma and Workman, 2011). Growing evidence has shown that histone PTMs influence transcription, DNA repair, replication and recombination (Bannister and Kouzarides, 2011).

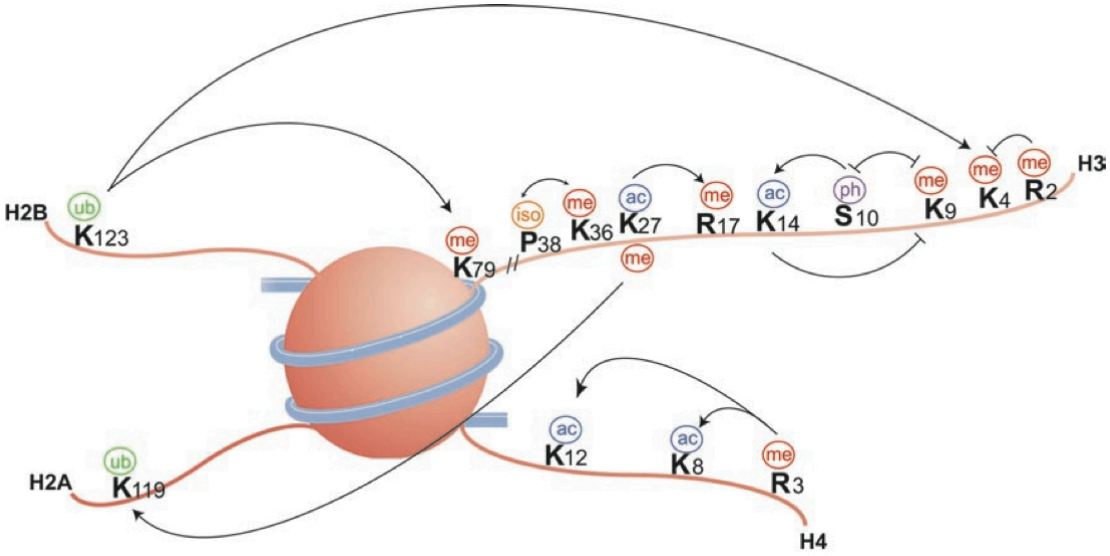


FIGURE 3. A few of the many PTMs that occur on histone tails. Some of these modifications include ubiquitylation (ub), methylation (me), acetylation (ac), phosphorylation (ph), and isomerization (iso). A PTM on one histone can influence a PTM modification on the same histone (cis-tail crosstalk) or on a different histone (trans-tail crosstalk). Positive influences between modifications are shown with arrowheads, and negative influences between modifications are shown with flatheads. K = lysine, P = proline, R = arginine, S = serine. Figure adapted from Bannister and Kouzarides 2011.

1.2.1.1 Histone Ubiquitylation

Among all the different types of histone PTMs, histone ubiquitylation is unique for a variety of reasons. First, it involves the covalent attachment of an 8.5 kilodalton (kDa)

polypeptide (ubiquitin) to specific lysine residues (Cole et al., 2015). This is different from the majority of other histone PTMs, which involve the covalent attachments of smaller entities like methyl and phosphate groups. Second, three different types of enzymes are involved in catalyzing the addition of ubiquitin to a lysine residue: an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin-protein ligase. Third, ubiquitylation of proteins is a modification that usually signals proteins for degradation. Specifically, polyubiquitylation (the addition of multiple ubiquitin molecules linked together in a chain) is a marker for protein degradation (Welchman et al., 2005). However, histone ubiquitylation usually occurs in the form of monoubiquitylation (the addition of a single ubiquitin molecule to a lysine residue), which is associated with signal transduction rather than protein degradation (Bannister and Kouzarides, 2011).

1.2.1.1.2 Monoubiquitylation of H2A.X

All H2A family members are subjected to monoubiquitylation. This family includes the canonical H2A and multiple variants such as H2A.Z, H2A.X, macroH2A and H2A.Bbd. Like most histone variants, H2A.X shares a similar amino acid sequence to its major histone counterpart (82% sequence similarity) (Chakravarthy et al., 2004). Additionally, H2A.X contains an extended C-terminus compared to H2A. **(Figure 4)**. H2A.X comprises about 10% of total H2A-type histones in cells (Pinto and Flaus, 2010). There are two distinct sites of monoubiquitylation on H2A.X: one on lysines 13/15 (K13/15) at the N-terminus (Mattiroli et al., 2012), and the other on lysines 118/119 (K118/119) at the C-terminus (lysines 118/119 have also been referred to as lysines 119/120, if the N-terminal methionine that gets cleaved from H2A.X

is counted) (Pan et al., 2011). It has been reported that both sites of monoubiquitylation play significant signaling roles in the repair of DNA DSBs (Mattiroli et al., 2012; Pan et al., 2012).

FIGURE 4. A comparison of amino acid sequences between canonical H2A histones and the histone variant H2A.X. Canonical H2A and H2A.X histones share 82% amino acid sequence similarity. The majority of the differences stem from the C-terminal ends of both. Figure adapted from Pinto and Flaus 2010.

1.3 The DNA Damage Response for Repair of DSBs

1.3.1 Mechanisms for DSB Repair

When a DSB occurs in the cell, there are multiple mechanisms available for its repair. Two of the most common and well-known mechanisms are non-homologous end joining (NHEJ) **(Figure 5A)** and homologous recombination (HR) **(Figure 5B)**. NHEJ can be active at any point in the cell cycle, but usually repairs DSBs during G0, G1 or G2 phase. In NHEJ, the

heterodimers Ku70/Ku80 bind to both ends of the DSB to recruit DNA-dependent protein kinase (DNA-PK). DNA-PK then recruits the exonuclease Artemis, which can remove one to four nucleotides at the ends of the break to create blunt ends for DNA ligase IV to ligate the ends together. The advantage of this mechanism is that it repairs DSBs in a quick manner; however, due to the possibility of nucleotide excision by Artemis, deletions can result. Unlike NHEJ, cells can only carry out DNA repair by HR in S and G2 phases because it requires the presence of a sister chromatid. For HR to take place, DNA ends need to be resected by the MRE11-RAD50-NBS1 (MRN) complex to generate 3' single-strand overhangs on each end. The recombinase RAD51 then helps the broken DNA strands invade the intact strands on the sister chromatid and use it as a template for repair. Although error-prone polymerases are used to facilitate the repair, HR is usually error-free (Ceccaldi et al, 2016; Li and Xu, 2016). In addition to NHEJ and HR, two other DNA repair mechanisms exist, although they are not as common and well-characterized. These are: single-strand annealing (SSA) **(Figure 5C)** and alternative end-joining (alt-EJ) **(Figure 5D)**. Both can only occur in the S and G2 phases of the cell cycle, and require end resection at the ends of the break like HR does. In SSA, interspersed long homologous regions (nucleotide repeats) between the two strands anneal together, assisted by Replication Protein A and RAD52. DNA ligase I joins the ends together. The problem with SSA is that the sequence between the long homologous regions that anneal together always becomes deleted in the final repaired product. The mechanism of alt-EJ is similar to that of SSA, except short homologous regions between the two strands anneal together, assisted by Poly(ADP-Ribose) Polymerase I (PARP1). DNA ligase III ligates the ends together. Deletions and/or insertions result from this mechanism, and it has been reported that chromosomal translocations can result as well if short homologous regions on DSBs in different chromosomes anneal together. Given

the mutagenic nature of SSA and alt-EJ, cells generally prefer to repair DSBs using NHEJ and HR. SSA and alt-EJ are only used when there are defects in the HR repair machinery (Ceccaldi et al., 2016).

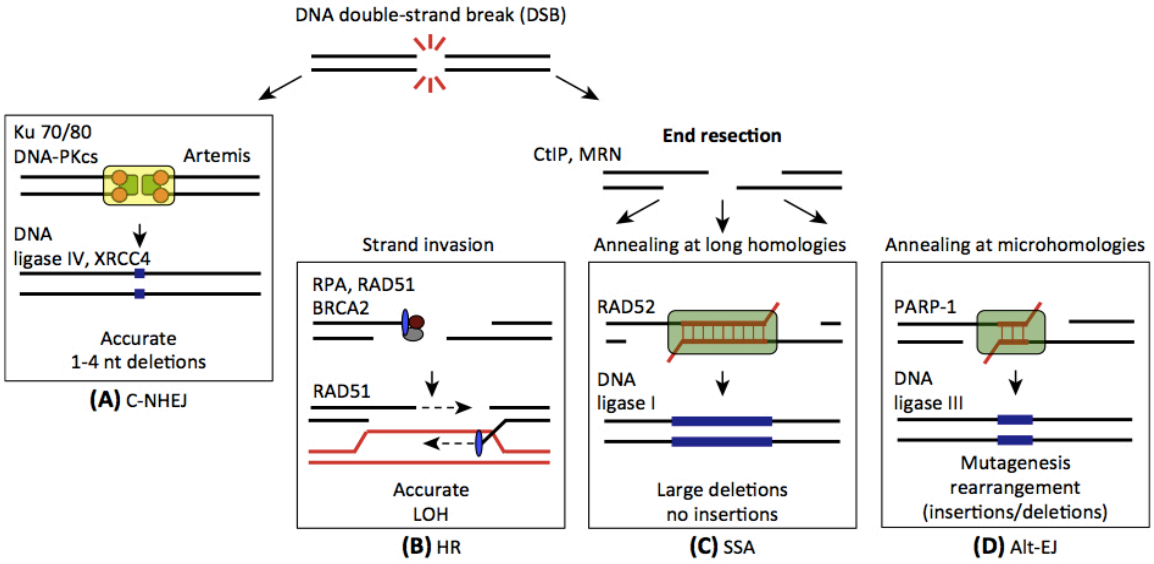


FIGURE 5. Schematic of the four possible DSB repair mechanisms. **A)** In NHEJ, the Ku70/80 heterodimers bind to the DNA ends to recruit the DNA-PK kinase, which in turn recruits the exonuclease Artemis. At some DSBs, Artemis removes some nucleotides at the ends of the break in order to facilitate efficient re-joining by DNA ligase IV with the help of XRCC4. Consequently, small deletions of 1-4 nucleotides can result. **B)** Before HR is initiated, both ends of the DNA break need to be resected. The exonuclease MRN, with the help of CtIP, cleaves a few nucleotides off each end, generating single-strand overhangs. Then, the recombinase RAD51, with the help of RPA and BRCA2, helps the broken DNA strands invade the intact strands of a homologous sister chromatid to use it as a template for repair. This usually results in accurate DNA repair, however loss of heterozygosity results. **C)** In SSA, resected DNA ends of the break anneal together at long homologous regions that were initially far apart from each other in the unbroken DNA strands. RAD52 assists in this process, and DNA ligase I helps ligate the ends together. Large deletions always result in the final repaired product. **D)** Alt-EJ is similar to SSA, in that resected DNA ends of the break anneal together at homologous regions. However, the homologous regions are much smaller. PARP1 assists with this process, and DNA ligase III ligates the ends together. Insertions and/or deletions result. Figure adapted from Ceccaldi et al. 2016.

Given the options of multiple DNA repair mechanisms, when a DSB occurs, a cell needs to decide which repair mechanism is most appropriate to use. As mentioned previously, the action of specific repair mechanisms largely depends on the cell cycle phase at the time of the DSB occurrence. When the cell is in G0 or G1 phase, the only mechanism available is NHEJ, whereas when the cell is in S or G2 phase, it prefers to use HR. In S and G2 phases, cyclin-dependent kinases (CDKs) phosphorylate proteins that promote end resection for initiation of HR. In particular, the CDKs phosphorylate the exonuclease Exo1 as well as CTBP interacting protein (CtIP). CtIP interacts with Breast Cancer Type 1 Susceptibility protein (BRCA1), which is an important factor for initiating HR (Ceccaldi et al., 2016). In contrast, p53-binding protein 1 (53BP1) is important for the initiation of NHEJ (Li and Xu, 2016). In G0 and G1 phases, 53BP1 is more abundant, and it becomes phosphorylated by Ataxia Telangiectasia Mutated (ATM) and/or other related kinases to recruit factors involved in NHEJ, such as Replication Timing Regulatory Factor 1 (Rif1), Pax Interacting Protein 1 (PTIP), and Artemis (Ceccaldi et al., 2016). Factors specific to NHEJ can actually antagonize factors specific to HR, and vice versa. For example, during NHEJ, 53BP1 and Rif1 prevent BRCA1 and CtIP from resecting the DNA ends for HR. Similarly, during HR, BRCA1 and CtIP prevent 53BP1 and Rif1 from accessing the break to initiate NHEJ (Li and Xu, 2016). Therefore, it is evident that cells have regulatory mechanisms in place to assist the cell in choosing the best possible DNA repair mechanism to initiate in the face of a DSB.

1.3.2 Phosphorylation of H2A.X at S139

While DNA repair mechanisms like NHEJ and HR are initiated in response to DSBs, the cell also needs a way of detecting the presence and location of DSBs so that the appropriate

repair machinery can assemble at the breaks. Indeed, a cascade of signaling events exists to communicate the formation of DSBs to the repair machineries. One early signaling event that takes place when a DSB is formed is the phosphorylation of H2A.X **(Figure 6A)**. The site of phosphorylation is at serine 139 (S139), which is located on the extended C-terminal tail unique to H2A.X. H2A.X phosphorylation (also referred to as γ-H2A.X) in response to DSBs formed from ionizing radiation (IR) was first discovered in 1998 by Bonner and colleagues (Rogaku et al., 1998). Since then, H2A.X phosphorylation has been widely accepted as a universal marker for sites of DSBs in cells. H2A.X is predominantly phosphorylated by the ATM kinase, although it can also be phosphorylated by the Ataxia Telangiectasia and Rad3 Related (ATR) kinase, or DNA-PKs. The MRN DSB recognition complex is responsible for recruiting ATM to DSBs and activating it. ATM exists as a dimer in its inactivated state, and once activated, it autophosphorylates to dissociate its dimeric state (Firsanov et al., 2011). In 2003, Elledge and colleagues discovered a protein that binds to phosphorylated H2A.X to help recruit DNA repair proteins to the site of breakage. They termed this protein Mediator of DNA Damage Checkpoint protein 1 (MDC1). Once MDC1 is recruited, it becomes phosphorylated by ATM as well (Stewart et al., 2003). MDC1 helps recruit additional MRN complexes, which in turn recruit additional ATM kinases to phosphorylate more H2A.X histones near the damaged site. This creates a positive feedback loop where the γ-H2A.X signal spreads in both directions away from the DSB (Lin et al., 2015), and this can occur up to megabases from both ends (Firsanov et al., 2011) **(Figure 6B)**. This amplification of γ-H2A.X ensures a sufficient amount of DNA repair proteins gets recruited to the break for initiation of repair. MDC1, through downstream signaling events, recruits 53BP1 and BRCA1, which are important initiators of NHEJ and HR, respectively (Lin et al., 2015). Once the lesion is repaired, H2A.X becomes de-phosphorylated by protein

phosphatase 4 (PP4) (Chowdhury et al. 2008), wild-type p53-induced phosphatase 1 (Wip1) (Macůrek et al., 2010), or protein phosphatase 6 (PP6) (Douglas et al., 2010). Although H2A.X phosphorylation has been well known and characterized within the field of DSB repair for many years, H2A.X monoubiquitylation at K118/119 and K13/15 are PTMs that have been more recently identified to play roles in the repair pathway (Mattiroli et al., 2012; Pan et al., 2012).

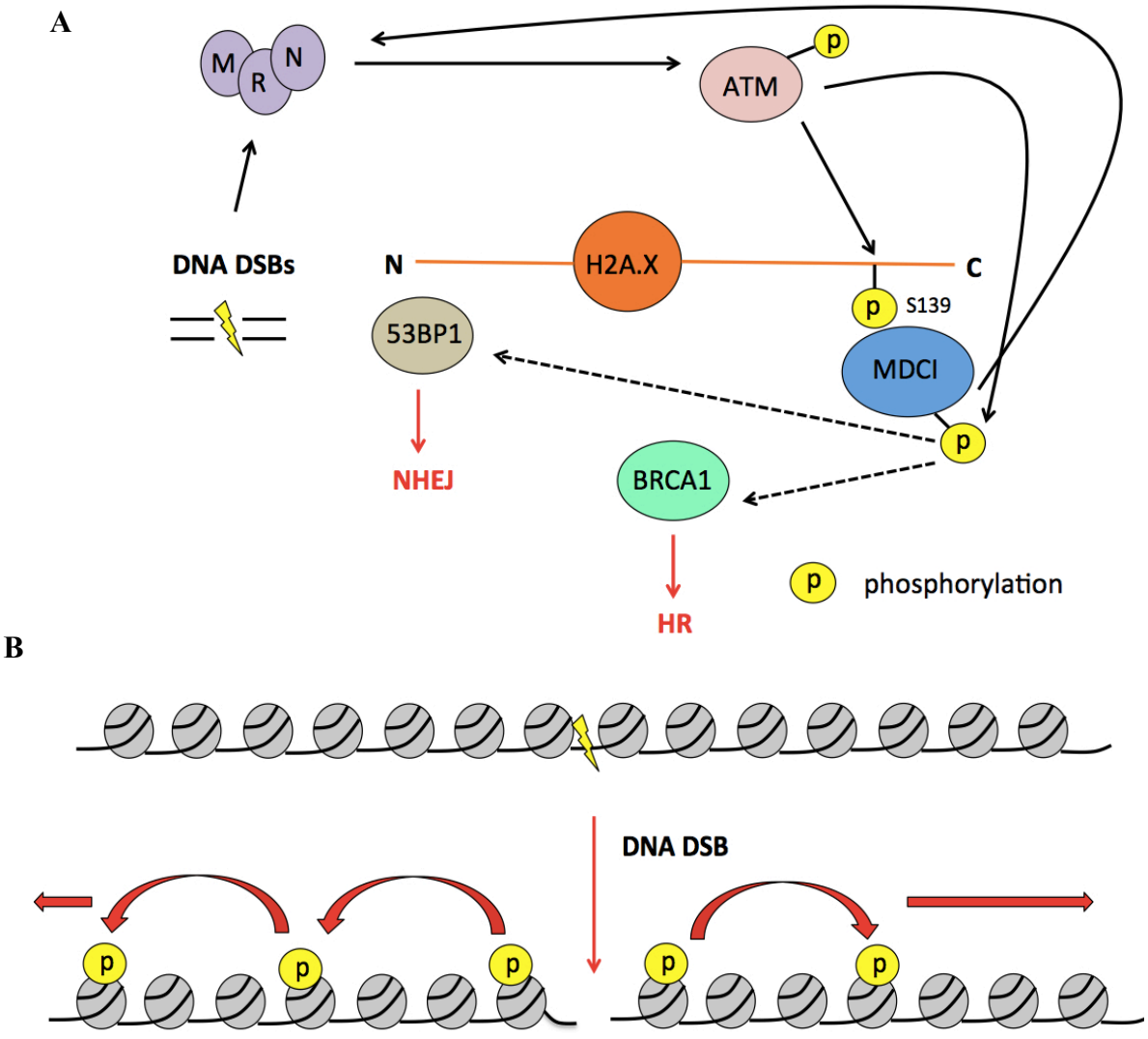


FIGURE 6. Mechanisms surrounding H2A.X S139 phosphorylation during DNA DSB repair. A) When DNA DSBs occur, the MRN complex localizes to DSBs, where it recruits the ATM kinase that is responsible for phosphorylating H2A.X at S139 on its C-terminal tail. MDC1 binds to phosphorylated H2A.X, where it also becomes phosphorylated by ATM. MDC1 creates a positive feedback loop where it recruits more MRN complexes to recruit more ATM kinases, thus spreading the H2A.X phosphorylation signal on both sides of the DSB. Phosphorylated MDC1 indirectly recruits 53BP1 and BRCA1, which are important for initiation of NHEJ and HR DNA repair mechanisms, respectively. **B)** A visual representation of the spreading of H2A.X S139 phosphorylation on both sides of the DSB. The phosphorylation signal can spread up to megabases away from the initial site. This ensures that a sufficient amount of DNA repair proteins get recruited to the break for initiation of repair.

1.3.3 Monoubiquitylation of H2A.X at K118/119

Although monoubiquitylation of H2A.X can occur on either K118 or K119, it has been reported that K119 is the dominant monoubiquitylation site, and monoubiquitylation at K118 only occurs if there is a mutation at K119 (Mattiroli et al., 2012). In response to DSBs, H2A.X becomes monoubiquitylated on K118/119 by the Ring Finger Protein 2 (RNF2) / BMI1complex (Pan et al., 2011; Wu et al., 2011) **(Figure 7)**. RNF2 (also known as RING1B) is the E3 ubiquitin ligase, and BMI1 is an enhancer of RNF2 (Pan et al., 2011). Some studies have proposed that monoubiquitylation at this site acts as a prerequisite for H2A.X phosphorylation at S139 (Pan et al., 2011; Wu et al., 2011). In particular, it has been suggested that this ubiquitylation mark acts as a 'docking' site to recruit the ATM kinase that phosphorylates H2A.X, leading to the eventual recruitment of DNA repair factors for NHEJ or HR. Interestingly, dimethylation of H2A.X at lysine 134 has also been suggested to help recruit the ATM kinase for H2A.X phosphorylation (Sone et al., 2014). After the DSB has been repaired, H2A.X becomes deubiquitylated at the K118/119 site by Ubiquitin Specific Peptidase 3 (USP3) (Schwertman et al., 2016).

Monoubiquitylation at K118/119 also occurs on canonical H2A in the absence of DNA damage: in fact, it has been known for some time that H2A monoubiquitylation at K118/119 is a modification involved in transcriptional repression of genes. H2A monoubiquitylation is also mediated by the RNF2/BMI1 complex, which is part of the Polycomb Repressive Complex 1 (PCR1) (Wu et al., 2011). Studies have suggested that H2A monoubiquitylation at K118/119 occurs at sites of DSBs to prevent transcription of genes around the break, since transcription at a DSB could interfere with the DNA repair process, and in turn threaten genomic integrity (Lin et

al., 2015). H2A.X is also known to be monoubiquitylated at K118/119 in the absence of DNA damage, where it likely plays a role in transcriptional repression (Mattiroli et al., 2012).

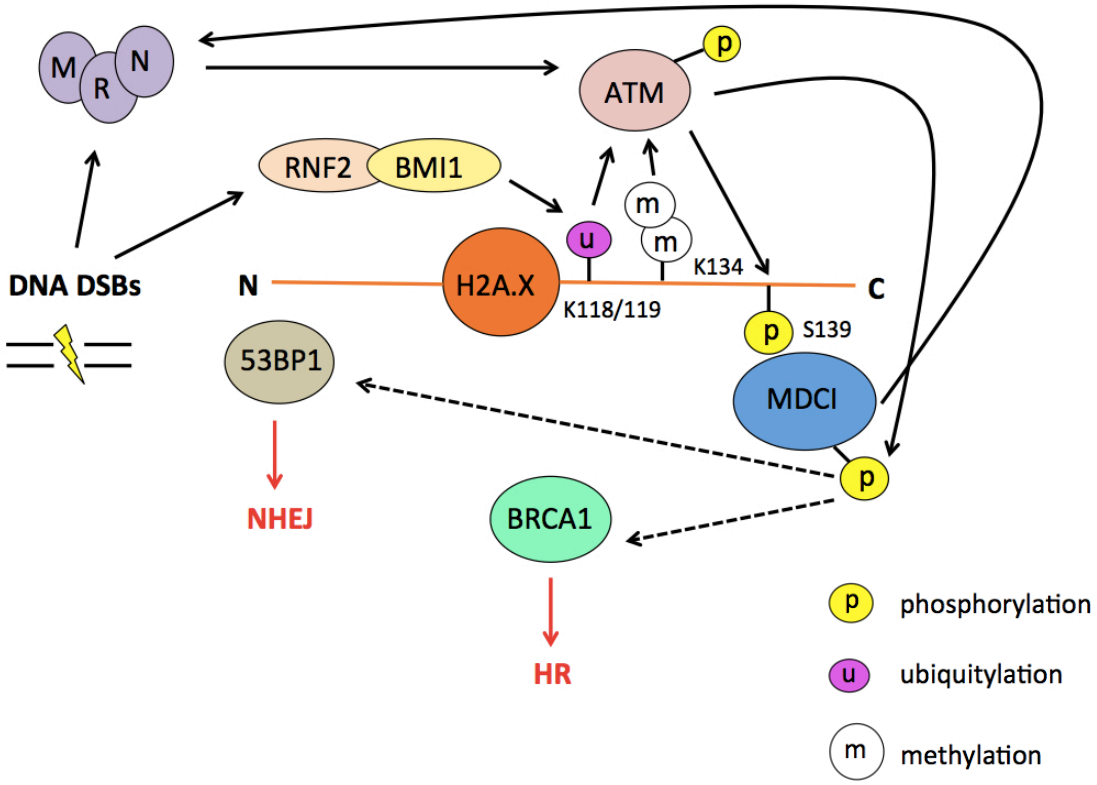


FIGURE 7. Mechanisms surrounding H2A.X monoubiquitylation at K118/119 during DNA DSB repair. Monoubiquitylation of H2A.X at K118/119 has been reported to act as a prerequisite for H2A.X phosphorylation, since it helps recruit the ATM kinase. The RNF2/BMI1 complex is responsible for this modification. Dimethylation of H2A.X at K134 has also been reported to help recruit ATM.

1.3.4 Monoubiquitylation of H2A.X/H2A at K13/15

H2A.X and canonical H2A can be monoubiquitylated on lysines 13 and 15; however, it is uncertain whether both these lysines can simultaneously be ubiquitylated, or if only one can be ubiquitylated at any given time. Unlike H2A.X monoubiquitylation at K118/119, H2A.X/H2A

monoubiquitylation at K13/15 has been reported to occur after H2A.X phosphorylation **(Figure 8)**. When MDC1 binds to the phosphorylated H2A.X, it also gets phosphorylated by ATM to recruit the E3 ubiquitin ligase Ring Finger Protein 8 (RNF8) (Lin et al., 2015). RNF8 then polyubiquitylates other substrates with the help of the E2-conjugating enzyme Ubc13. RNF8 polyubiquitylates its substrates in a K63-linked manner, whereby each ubiquitin attaches to K63 on the next ubiquitin. One reported substrate of RNF8 polyubiquitylation activity is histone H1 (Thorslund et al., 2015). This polyubiquitylation of RNF8-targets leads to the recruitment of a second E3 ubiquitin ligase, Ring Finger Protein 168 (RNF168), which is the E3 ligase responsible for monoubiquitylation of H2A.X/H2A at K13/15 (Mattiroli et al., 2012). H4 histones near DSBs become neddylated (modified by NEDD8, a ubiquitin-like modifier), which also assists with RNF168 recruitment (Ma et al., 2013). After repair, H2A.X/H2A becomes deubiquitylated at the K13/15 site by Ubiquitin Specific Peptidase 51, 3 or 16 (USP51, USP3 or USP16) (Wang et al., 2016).

H2A.X/H2A monoubiquitylation at K13/15 has been reported to be responsible for the recruitment of 53BP1 and BRCA1 for NHEJ and HR, respectively **(Figure 8)**. Interestingly, 53BP1 has been shown to only be recruited to H2A.X/H2A if monoubiquitylation occurs on K15, and if dimethylation occurs on histone H4 at K20 or histone H3 at K79. Indeed, 53BP1 contains a ubiquitin-binding domain and a Tudor domain that allows it to simultaneously bind to H2A.X/H2A ubiquitylation at K15 and H4 dimethylation at K20 / H3 dimethylation at K79 (Kocyłowski et al., 2015). The monoubiquitylation marks on K13/15 can be extended to form K63-linked polyubiquitin chains on H2A.X/H2A. RNF8 has been suggested to be responsible for ubiquitin chain extension on K13/15. Polyubiquitin chains on H2A.X/H2A are important for the recruitment of Receptor-Associated Protein 80 (RAP80) (Mattiroli et al., 2012). RAP80 in

turn recruits BRCA1 (Gatti et al., 2012). BRCA1 also has E3 ligase activity, and it may ubiquitylate proteins at sites of DNA damage (Vissers et al., 2008). Additionally, RNF168 has been reported to form K27-linked polyubiquitin chains on proteins in the surrounding chromatin as well (Dantuma and Attikum 2015). It is thought that ubiquitylation events can change the conformation of chromatin at the site of damage, making it more open and accessible to repair factors, as opposed to just acting as recruitment centers for proteins (Mattiroli et al., 2012). Finally, many proteins involved in DNA repair signaling, such as MDC1, 53BP1, BRCA1, RNF168, RAP80 and BMI1 are also substrates of SUMOylation (Schwertman et al., 2016), which involves the covalent attachment of the SUMO (small ubiquitin-like modifier) moiety to lysine residues on a protein (Hendriks et al., 2015). SUMOylation has been reported to have different functions, including mediation of protein-protein interactions and regulation of enzyme activity. SUMOylated proteins are often modified by ubiquitin as well (Schwertman et al., 2016).

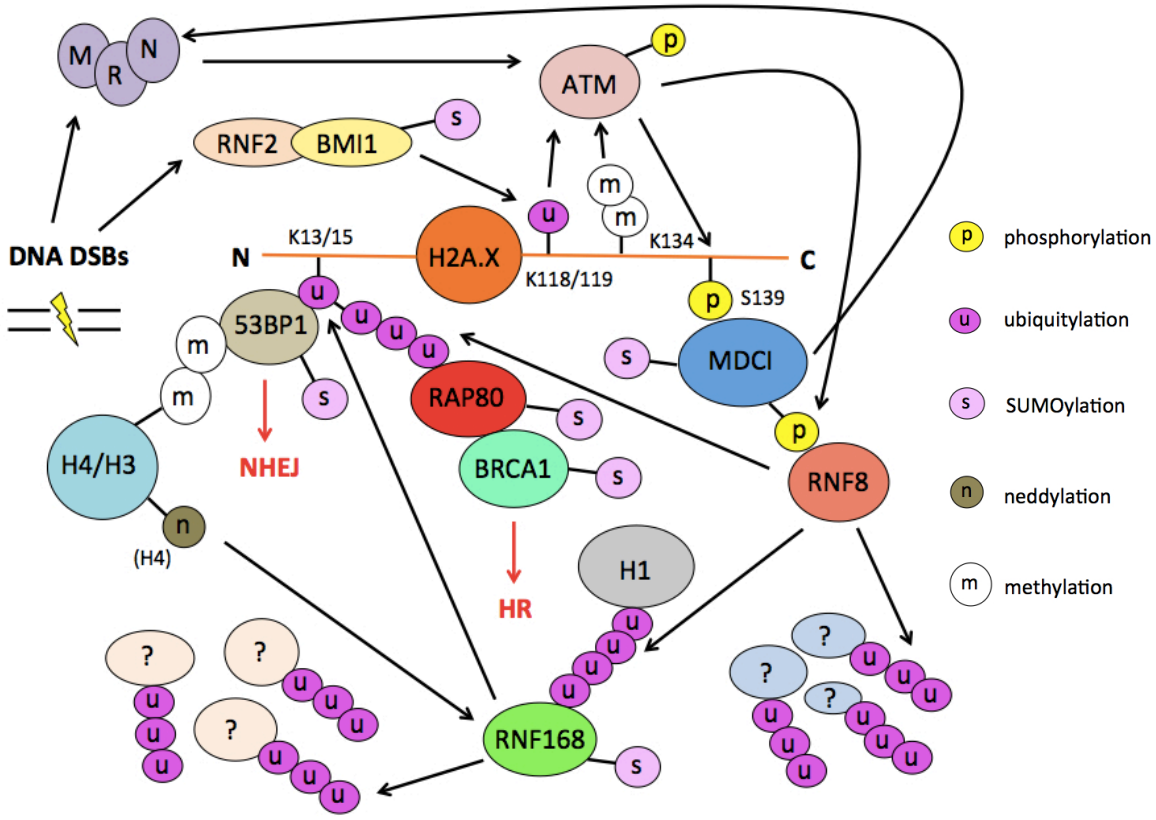


FIGURE 8. Mechanisms surrounding H2A.X monoubiquitylation at K13/15 during DNA DSB repair. A second monoubquitylation mark on H2A.X, at K13/15 on the N-terminal tail, occurs downstream of H2A.X phosphorylation. The RNF8 E3 ubiquitin ligase binds to phosphorylated MDC1, where it polyubiquitylates proteins like histone H1 to recruit a second E3 ubiquitin ligase, RNF168. RNF168 monoubiquitylates H2A.X at K13/15, and has been reported to polyubiquitylate other proteins. 53BP1 binds to monoubiquitylated H2A.X at K15 and dimethylated H4 at K20 / H3 at K79. RNF8 has been suggested to polyubiquitylate H2A.X at K13/15 to recruit RAP80, which recruits BRCA1. Other protein PTMs are known to occur in response to DSBs as well. For example, H4 becomes neddylated to help recruit RNF168. Other proteins can become SUMOylated, which can enhance enzyme activity or promote protein-protein interactions. Note: Canonical H2A histones can also become monoubiquitylated/polyubiquitylated at K13/15 in response to DSBs to recruit 53BP1 and BRCA1.

1.4 Objectives of Project

Evidently, much information has been acquired regarding the mechanisms that surround H2A.X monoubiquitylation at K118/119 and K13/15 during DNA DSB repair signaling. Over time, more and more proteins and PTMs have been discovered to play a role in this complex and intricate signaling network. However, there are still questions that remain and aspects of the pathway that need further clarification. For example, how does the RNF2/BMI1 complex become recruited to sites of DSBs? Besides H1, are there any other targets that become polyubiquitylated by RNF8 to recruit RNF168? What are the substrates of RNF168's polyubiquitylation activity in the chromatin surrounding DSBs? Because signaling events on one histone are known to influence downstream events on other histones, H2A.X ubiquitylation may also promote protein recruitments and PTMs on the other core histones as well. Noticeably, there are still proteins involved in this signaling network that have yet to be discovered. In addition, the exact relationship between H2A.X monoubiquitylation at K118/119, H2A.X phosphorylation at S139, and H2A.X monoubiquitylation at K13/15 may not be as clear as the literature suggests. Although it may seem that H2A.X monoubiquitylation at K118/119 is required for H2A.X phosphorylation, which in turn is required for H2A.X monoubiquitylation at K13/15, the sequence of events may not be strictly linear. The main objective of this project is to gain a better understanding of the mechanisms associated with H2A.X monoubiquitylation marks in the DNA damage response pathway. This project primarily concerns itself with two questions: 1) What proteins are associated with ubiquitylated H2A.X - containing nucleosomes in the context of DNA damage?, and 2) What is the relationship between H2A.X monoubiquitylation at K118/119, H2A.X monoubiquitylation at K13/15, and H2A.X

phosphorylation at S139 during DNA damage? The experimental approaches that have been taken to answer these questions will be discussed.

1.5 Experimental Approaches

1.5.1 Identifying Proteins that Interact with Ubiquitylated H2A.X - Containing Nucleosomes in a DNA Damage-Dependent Manner

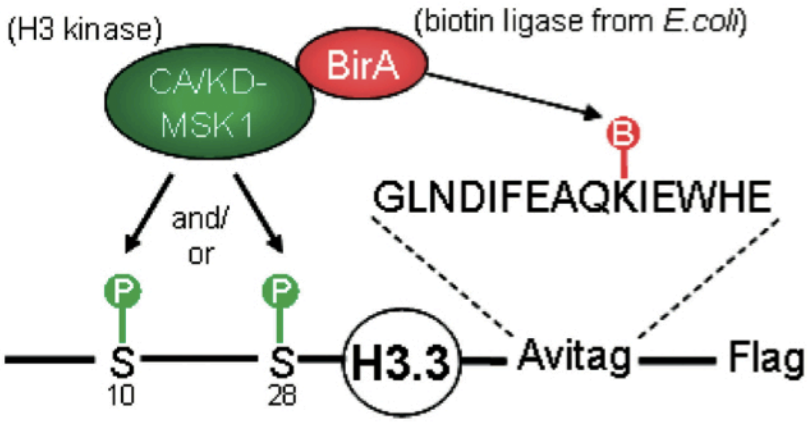
In order to identify novel proteins that interact with ubiquitylated H2A.X-containing nucleosomes, we have begun to develop and test a novel purification system for the isolation of ubiquitylated H2A.X-containing nucleosomes. This purification system is based on an original nucleosome purification method termed BICON (**B**iotinylation-assisted **I**solation of **CO**-modified **N**ucleosomes), which was previously developed by Lau and Cheung (Lau and Cheung, 2012) **(Figure 9A)**. The BICON technique was developed to purify nucleosomes that contained phosphorylated H3.3 (a variant of histone H3), that were specifically phosphorylated at S10 and/or S28 by Mitogen and Stress-Activated Protein Kinase-1 (MSK1). As part of the approach, modified MSK1 and H3.3 constructs were developed. In the MSK1 construct, the *Escherichia coli* (*E. coli*) biotin ligase BirA was fused to the C-terminus of MSK1. BirA is an enzyme that catalyzes the covalent attachment of biotin (a small organic molecule) to a specific lysine residue within a unique 15 amino acid sequence 'GLNDIFEAQ**K**IEWHE'. This unique sequence is commonly referred to as an Avi-tag, and is not found within any mammalian proteins. The H3.3 construct was engineered to have this unique Avi-tag at its C-terminus. In cells transfected with both constructs, when the MSK1-BirA fusion protein phosphorylates the Avi-tagged H3.3, the

BirA portion of the fusion enzyme would biotinylate the Avi-tag due to their close proximity. This system couples biotinylation and phosphorylation of H3.3. Therefore, by purifying nucleosomes containing biotinylated H3.3, these nucleosomes will also be phosphorylated by MSK1 (hence the name **B**iotinylation-assisted **I**solation of **CO**-modified **N**ucleosomes). Given the ease of purifying biotinylated proteins by streptavidin-coupled beads, this method allows for high efficiency of purifying MSK1-phosphorylated H3.3.

We applied the main principles of the BICON method toward the development of a system that could be used to purify ubiquitylated H2A.X-containing nucleosomes **(Figure 9B)**. This approach involves the creation of an H2A.X-BirA fusion protein where the BirA enzyme is fused to the C-terminus of H2A.X, as well as ubiquitin molecules engineered to contain the Avi-tag at its N-terminus. Co-expression of these two components in mammalian cells would result in H2A.X-BirA being incorporated into nucleosomes and chromatin, and then ubiquitylated with either endogenous or Avi-tagged ubiquitin. The idea is that when the H2A.X-BirA is Avi-ubiqutiylated, the BirA portion of the protein would preferentially biotinylate the Avi-ubiquitin attached to itself due to the proximity of the enzyme and target, and also because of the increased efficiencies of an intramolecular reaction. After allowing the biotinylated/ubiquitylated H2A.X-BirA to accumulate over 48 hours post transfection, we would lyse the transfected cells and harvest mononucleosomes (individual nucleosomes) from the lysates that will be used as input for pull-down assays. Nucleosomes containing ubiquitylated and biotinylated H2A.X-BirA can be pulled down using streptavidin-coupled beads, and co-purifying proteins can be eluted and identified by mass spectrometry. To distinguish between proteins that associate with ubiquitylated H2A.X-BirA - containing nucleosomes in a DNA damage - dependent manner as opposed to a non DNA damage - dependent manner, streptavidin pull-downs can be done with

DNA damaged and non-DNA damaged cells. Any proteins found to be specific to DNA damage - induced samples would provide more insight into the mechanisms associated with ubiquitylation of H2A.X histones in the DNA damage response. It should be noted that this purification approach has already been utilized in our lab for the isolation of ubiquitylated H2A.Z – containing nucleosomes, involving an H2A.Z-BirA fusion protein and Avi-tagged ubiquitin in a similar manner.

A


(H3 kinase)
(biotin ligase from *E.coli*)
CA/KD-MSK1
BirA
B
GLNDIFEAQKIEWHE
and/or
P
P
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10
S
28
H3.3
Avitag
Flag

B

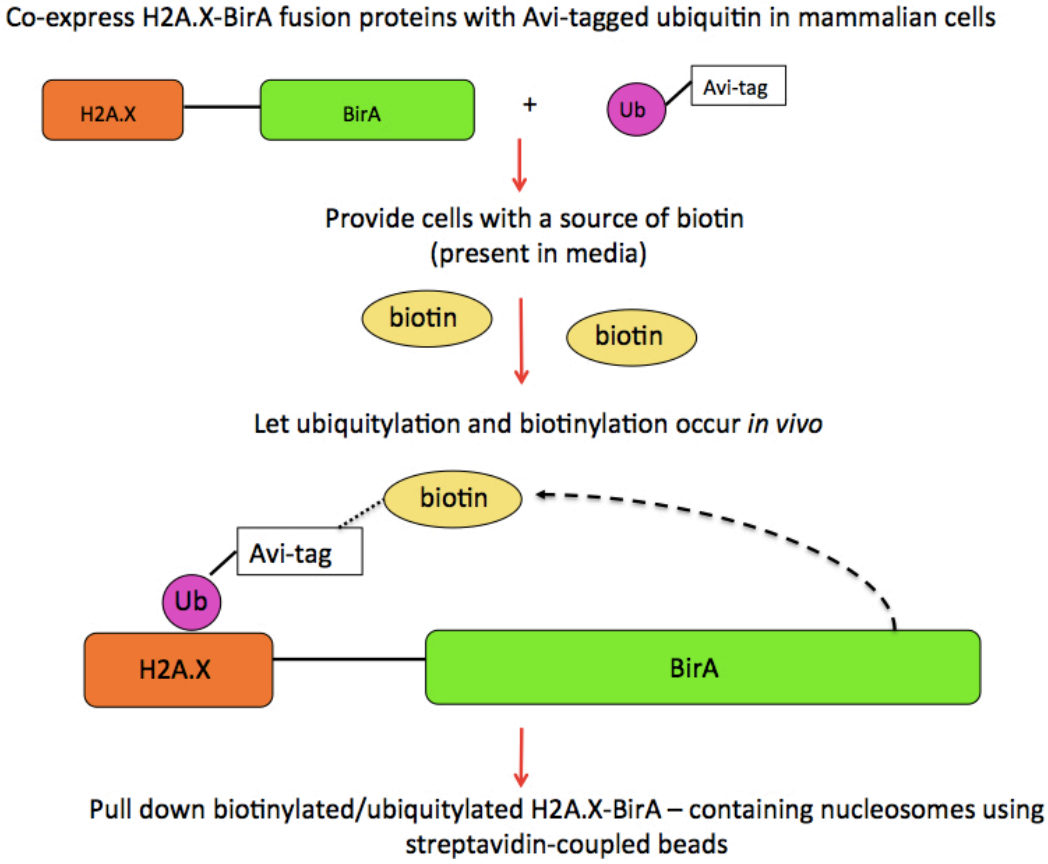

Co-express H2A.X-BirA fusion proteins with Avi-tagged ubiquitin in mammalian cells
H2A.X
BirA
+
Ub
Avi-tag
Provide cells with a source of biotin
(present in media)
biotin
biotin
Let ubiquitylation and biotinylation occur *in vivo*
biotin
Avi-tag
Ub
H2A.X
BirA
Pull down biotinylated/ubiquitylated H2A.X-BirA – containing nucleosomes using
streptavidin-coupled beads

FIGURE 9. Two different histone purification systems utilizing BirA-fusion proteins. A) A schematic of the original BICON (**B**iotinylation-assisted **I**solation of **CO**-modified **N**ucleosomes) method developed by Lau and Cheung. This method was developed to purify nucleosomes that contained histone H3.3 that was specifically phosphorylated by MSK1. This method involved the creation of an MSK1-BirA fusion protein and an H3.3 histone with an Avi-tag. BirA is a biotin ligase from *E. coli* that can catalyze the addition of biotin to a specific lysine residue within the Avi-tag, a unique 15 amino acid sequence that is not present within any mammalian proteins. They reasoned that, if the MSK1-BirA protein phosphorylated H3.3, BirA could biotinylate the Avi-tag on H3.3 due to their close proximity. Biotinylated/phosphorylated H3.3 – containing nucleosomes could then be purified in pull-downs using streptavidin-coupled beads, which have very high affinity for biotin. Figure adapted from Lau and Cheung 2012. **B)** A schematic of the system we have begun to develop and test for the isolation of ubiquitylated H2A.X-containing nucleosomes. This purification system would involve the creation of an H2A.X-BirA fusion protein and ubiquitin with an Avi-tag. First, we would co-express H2A.X-BirA and Avi-tagged ubiquitin in mammalian cells, and provide cells with a sufficient amount of biotin from the cell culture media. If H2A.X-BirA becomes ubiquitylated with Avi-tagged ubiquitin, the BirA segment should be able to 'flip' over and biotinylate the Avi-tag due to its substrate specificity and their close proximity to each other. In this manner, ubiquitylated H2A.X-BirA-containing nucleosomes could be marked with biotin, and subsequently purified using streptavidin-coupled beads.

1.5.2 Elucidating the Relationship Between H2A.X Monoubiquitylation at K118/119, H2A.X Monoubiquitylation at K13/15 and H2A.X Phosphorylation at S139 During DNA Damage

To gain a better understanding of the relationship between H2A.X monoubiquitylation at K118/119, H2A.X monoubiquitylation at K13/15, and H2A.X phosphorylation at S139 during DNA damage, we wanted to engineer different H2A.X-BirA constructs to contain mutations at each of these sites of PTM. Therefore, the following constructs were desired: H2A.X-K118/119R (arginine)-BirA, H2A.X-K13/15R-BirA, H2A.X-K118/119R, K13/15R-BirA, and H2A.X-S139A (alanine)-BirA. These mutant H2A.X-BirA proteins would be expressed individually in cells, DNA damage would be induced, and then their ubiquitylation and phosphorylation levels would be analyzed by Western-blotting nuclear lysates from each sample

with the appropriate antibodies. By collectively evaluating changes in the ubiquitylation and phosphorylation levels associated with each mutant H2A.X-BirA protein, the relationship among these PTMs during DNA damage may become clearer. Additionally, the mutant H2A.X-BirA – containing nucleosomes could be purified by immunoprecipitation with the appropriate antibodies, and then differences in the recruitment of specific DDR proteins could be analyzed by Western-blotting eluted proteins with antibodies against proteins of interest. For example, if the results show a loss of MDC1 recruitment with H2A.X-K118/119R-BirA, but no loss of MDC1 with H2A.X-K13/15R-BirA, this would suggest that H2A.X monoubiquitylation at K118/119 precedes phosphorylation, and H2A.X monoubiquitylation at K13/15 occurs after phosphorylation (since MDC1 is recruited to phosphorylated H2A.X).

1.6 Significance of Study

H2A.X monoubiquitylation marks help signal repair proteins to sites of DSBs. It is essential for all DSBs to be detected and repaired accordingly because if they are not, genomic instability can result, which often leads to the development of cancers (Pan et al., 2011). Therefore, it is important to study the mechanisms surrounding H2A.X monoubiquitylation marks because they provide a protective function against tumor formation. If a mutation were to occur in any protein that is associated with the formation or function of either monoubiquitylation mark, the DSB wouldn't be able to be repaired. Thus, it is advantageous to have more knowledge of the proteins and processes involved in the signaling process. New information gained may be useful for the development of future cancer therapies.

CHAPTER 2: MATERIALS AND METHODS

2.1 Plasmid Constructs

The H2A.X-BirA fusion protein was designed such that the human H2A.X histone is fused to the N-terminus of the bacterial *E. coli* biotin ligase BirA. In between the H2A.X and BirA segments is a short amino acid linker (GGGS), followed by a Flag-tag (DYKDDDDK), followed by a longer amino acid linker (GGGSGGGGT). The H2A.X-GGGS-Flag-GGGSGGGGT-BirA coding sequence was cloned into a pcDNA3.1+ vector. This H2A.X-Flag-BirA - expressing plasmid was derived from a pcDNA3.1+ plasmid expressing H2A.Z-GGGS-Flag-GGGSGGGGT-BirA: the H2A.X gene was amplified by polymerase chain reaction (PCR) and sub-cloned into digested H2A.Z-Flag-BirA plasmid to replace the H2A.Z gene. Plasmids expressing mutant forms of H2A.X-Flag-BirA (H2A.X-K118/119R-Flag-BirA, H2A.X-K13/15R-Flag-BirA, H2A.X-K118/119R, K15/15R-Flag-BirA, and H2A.X-S139A-Flag-BirA) were all derived from the original pcDNA3.1+ plasmid expressing H2A.X-Flag-BirA: the wild-type (WT) H2A.X gene was PCR-amplified with primers containing the appropriate mutations, and sub-cloned into digested H2A.X-Flag-BirA plasmid to replace the WT H2A.X gene.

The pSpCas9(BB)-2A-Puro (PX459 vector) V2.0 plasmid was purchased from AddGene (plasmid #62988): this plasmid expresses Cas9 from bacterial *Streptomyces pyogenes* and includes a cloning site for a subgenomic RNA (sgRNA). The sequence of a human Alu element (TGTAATCCCAGCACTTTGGG) was cloned into the sgRNA cloning site, which was used as the guide RNA sequence for Cas9. An MCSV (vector) plasmid expressing HA-tagged (YPYDVPDYA) mouse RNF168 was kindly provided by Dr. Razq Hakem (University of

Toronto). The following plasmids were pre-existent in the lab: pcDNA3.1+ expressing Avi-tagged (GLNDIFEAQKIEWHE) ubiquitin, and an empty pBABE vector.

2.2 Cell Culture and Transfections

293T human embryonic kidney cells, 293 human embryonic kidney cells and U20S human bone cancer cells were used in this study. 293T cells were used for most experiments due to their high proliferation rates and ability to express exogenous proteins at high levels. 293T and 293 cells were cultured in Dulbecco's Modified Eagle's Medium (GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (Wisent) and 1% penicillin/streptomycin (GE Healthcare Life Sciences). U2OS human bone cancer cells were cultured in McCoy's 5a Medium Modified (GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were incubated at 37°C with 5% CO_2.

Most transient transfections were done using Polyethylenimine (PEI) linear (Polysciences). Cells were seeded in Petri dishes the day prior to transfections at a density that would allow the cells to be ~ 50-70% confluent at the time of transfections. Three hours prior to transfections, old media from the dishes were removed and replaced with fresh media. At the time of transfections, plasmids were mixed with PEI in Opti-MEM Reduced Serum Medium (Gibco). The amount of plasmid, PEI and Opti-MEM media used was dependent on the size dish the cells were in: 1 μg of each plasmid in 0.5 ml Opti-MEM with 18 μg/ml PEI for 60 mm dishes, 3μg of each plasmid in 1 ml Opti-MEM with 18 μg/ml PEI for 100 mm dishes, and 6 μg of each plasmid in 1.5 ml Opti-MEM with 24 μg/ml PEI for 150 mm dishes. After all components were mixed, the transfection samples were incubated at room temperature (RT) for

20 minutes before gently being added dropwise to the cells. The transfected cells were then incubated at 37°C with 5% CO_2 for 2 days.

Some transfections were done using PolyJet In Vitro DNA Transfection Reagent (Signa Gen Laboratories) according to the manufacturer's instructions. The transfected cells were then incubated at 37°C with 5% CO_2 for 2 days.

2.3 Drug Treatments

The drugs etoposide (EI383), methyl methanesulfonate (MMS) (129925), doxorubicin hydrochloride (D1515), and neocarzinostatin (NCS) (N9162) used in this study were all purchased from Sigma. For experiments that exclusively consisted of untransfected cell samples, cells were treated with the appropriate drug at ~80-100% confluency. For experiments that consisted of transfected cell samples, cells were treated with the appropriate drug 2 days after transfections, at ~80-100% confluency. For all drug treatments, old media from the dishes were removed and replaced with fresh media containing the appropriate drug at the appropriate concentration (indicated). In some experiments, as a control for MMS (which was dissolved in sterile water), media from dishes were removed and replaced with media containing added sterile water. The volume of sterile water added to the media was equal to the volume of MMS added to the media for the MMS-treated sample(s) in the experiment. In some experiments, as a control for etoposide (which was dissolved in dimethyl sulfoxide, DMSO), media from dishes were removed and replaced with media containing added DMSO. The volume of DMSO added to the media was equal to the volume of etoposide added to the media for the etoposide-treated sample(s) in the experiment. Due to doxorubicin hydrochloride and NCS being light sensitive,

handling and treatment with these two drugs were done in minimized light. Cells were incubated with their respective drugs at 37°C with 5% CO_2 for various lengths of time (indicated).

2.4 Cell Harvesting and Preparation of Nuclear Lysates

All cells were harvested by scraping the cells off the bottom of the dishes while still in media. The scraped cells were centrifuged at 150 x g at 4°C for 5-15 minutes to pellet the cells. The supernatants were removed and the cells were washed by re-suspension in cold Phosphate Buffered Saline (PBS) without magnesium chloride (Wisent), and centrifuged at 150 x g at 4°C for 5-15 minutes. The supernatants were removed, and the cells were re-suspended in fresh cold PBS and centrifuged again at 150 x g at 4°C for 5-15 minutes. The supernatants were removed, and the cell pellets were kept on ice.

To prepare nuclear lysates in a manner that would denature proteins and abolish protein-protein interactions, the following steps were done. First, the cells were re-suspended in cold Nuclear Extraction Buffer (10 mM Tris pH 7.6, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.65% NP-40 with protease inhibitors and N-ethylmaleimide) and centrifuged at 922 x g at 4°C for 5 minutes. The supernatants (cytoplasmic fractions) were removed, and the nuclei were re-suspended in boiling 2X sodium dodecyl sulfate (SDS) lysis buffer (20 mM Tris pH 7.4, 20 mM EDTA, 2% SDS, 20% glycerol). The nuclei were then sonicated (duty cycle: 50, output control: 3, ~10 pulses) and immediately boiled for 5 minutes to denature the proteins. The nuclear lysates were then stored at -20°C.

To prepare nuclear lysates in a manner that would preserve protein-protein interactions, the following steps were done. First, cells were washed by re-suspension in cold Buffer A (10 mM HEPES pH 7.35, 10 mM KCl, 1.5 mM $MgCl_2$, 340 mM sucrose, 10% glycerol, 1 mM DTT

with protease inhibitors and N-ethylmaleimide). Cells were centrifuged at 300 x g at 4°C for 5 minutes, then the supernatants were removed and the cells were re-suspended in cold Buffer A + 0.2% Triton-X 100. The cells were incubated on ice for 5 minutes, and then centrifuged at 1300 x g at 4°C for 5 minutes to pellet the nuclei. The supernatants (cytoplasmic fractions) were removed, and the nuclei were re-suspended in cold E1A buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Tween-20 with protease inhibitors and N-ethylmaleimide). The re-suspended nuclei were then sonicated (duty cycle: 50, output control: 3, ~ 60-120 pulses in 10-pulse increments while samples were kept on ice in between increments). After sonication, the samples were centrifuged at 15.7 x g at 4°C for 5 minutes to pellet insoluble material. The supernatants (nuclear lysates) were collected and kept on ice until subjected to Flag immunoprecipitations or streptavidin pull-downs.

2.5 Preparation of Mononucleosomes with Micrococcal Nuclease (MNase)

Cells were harvested as previously described, and then cell pellets were washed by re-suspension in cold Buffer A. Cells were centrifuged at 300 x g at 4°C for 5 minutes, then the supernatants were removed and the cells were re-suspended in cold Buffer A + 0.2% Triton-X 100. Cells were incubated on ice for 5 minutes, and then centrifuged at 1300 x g at 4°C for 5 minutes to pellet the nuclei. The supernatants (cytoplasmic fractions) were removed, and the nuclei were re-suspended in Cutting Buffer (15 mM NaCl, 60 mM KCl, 10 mM Tris pH 7.5 with protease inhibitors and N-ethylmaleimide). The nuclei were centrifuged at 1300 x g at 4°C for 5 minutes, the supernatants were removed, and the nuclei were re-suspended in Cutting Buffer + 2 mM $CaCl_2$. To digest the chromatin to obtain mononucleosomes, MNase (Worthington) was added to the nuclei suspensions (1 U MNase/10^6 cells). The nuclei were incubated with MNase

at 37°C for 30 minutes, and then EGTA was added to a final concentration of 20 mM to stop MNase activity. Nuclei were centrifuged at 1300 x g at 4°C for 5 minutes. The supernatants (denoted as MNase fractions, which contained mononucleosomes that floated out of the nuclei) were removed and kept on ice. To lyse the nuclei, the nuclei were re-suspended in Tris-EDTA (TE) buffer (with protease inhibitors and N-ethylmaleimide) and incubated on ice for 30 minutes, while mixing every 10 minutes. To pellet cell debris and insoluble material, the samples were centrifuged at 15.7 x g at 4°C for 5 minutes. The supernatants (denoted as TE fractions, which contained the remainder of mononucleosomes and other soluble nuclear proteins) were removed and kept on ice. To re-adjust the salt concentrations of the MNase fractions (back to physiological concentrations), an equal volume of 2X Buffer E (30 mM Tris pH 7.5, 225 mM NaCl, 3 mM $MgCl_2$, 20% glycerol, 0.4% Triton-X 100 with protease inhibitors and N-ethylmaleimide) was slowly added to each fraction (dropwise with constant mixing on a vortexer at low speed). To re-adjust the salt concentrations of the TE fractions, 3X Buffer D (60 mM HEPES pH 7.5, 450 mM NaCl, 4.5 mM $MgCl_2$, 0.6 mM EGTA, 0.6 % Triton-X 100, 30% glycerol with protease inhibitors and N-ethylmaleimide) was slowly added to the TE fractions at a volume that was half that of the TE fractions (dropwise with constant mixing on a vortexer at low speed). To pellet precipitated material, the MNase and TE fractions were centrifuged at 15.7 x g at 4°C for 5 minutes. The supernatants were removed, and then the MNase and TE fractions were mixed together (for each sample). The combined MNase and TE fractions were kept on ice until subjected to Flag immunoprecipitations.

2.6 Flag Immunoprecipitations (IPs)

Anti-Flag M2 Affinity Gel agarose beads (Sigma) were used to immunoprecipitate Flag-tagged proteins. Before use, the bead slurry was washed with cold 1X Buffer D (20 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EGTA, 0.2 % Triton-X 100, 10% glycerol) and then centrifuged at 3000-7000 x g at 4°C for 30 seconds. The supernatants were removed, and the washes were repeated a second time. The beads were re-suspended in cold 1X Buffer D (with protease inhibitors and N-ethylmaleimide) at a volume equal to the initial amount of slurry. Prior to being mixed with the beads, nuclear lysates (denatured or non-denatured) or mononucleosomal extracts (combined MNase and TE fractions) were diluted in an appropriate volume of 1X Buffer D (with protease inhibitors and N-ethylmaleimide). A fraction of each sample (input) was collected and stored at -20°C. The remainder of the samples were mixed with the beads and left to tumble on a rotisserie at 4°C overnight. The following day, the samples were centrifuged at 3000-7000 x g at 4°C for 30 seconds. The supernatants (unbound fractions) were removed. The beads were washed with cold 1X Buffer D (with protease inhibitors and N-ethylmaleimide) and then centrifuged at 7000 x g at 4°C for 30 seconds. The supernatants were removed, and the washes were repeated three more times with 1X Buffer D (with protease inhibitors and N-ethylmaleimide) and 4 more times with 1X Buffer D + 0.5% Triton-X 100 (with protease inhibitors and N-ethylmaleimide). To elute bound proteins from the beads, the beads were re-suspended in 2X SDS lysis buffer and boiled for 5 minutes. The samples were centrifuged at 7000 x g at RT for 30 seconds, and the supernatants (bound fractions) were stored at -20°C.

2.7 Streptavidin Pull-Downs

Biotinylated proteins were isolated using Streptavidin Sepharose High Performance beads (GE Healthcare Life Sciences). Before use, the beads were washed with 1X Buffer D and then centrifuged at 3000-7000 x g at RT for 30 seconds. The supernatants were removed, and the washes were repeated a second time. The beads were re-suspended in 1X Buffer D (with protease inhibitors and N-ethylmaleimide) at a volume equal to the initial amount of slurry. Prior to being mixed with the beads, nuclear lysates (denatured or non-denatured) were diluted in an appropriate volume of 1X Buffer D (with protease inhibitors and N-ethylmaleimide). A fraction of each sample (input) was collected and stored at -20°C. The remainder of the samples were mixed with the beads and left to tumble on a rotisserie at RT for 1 hour or 4°C overnight. Afterwards, the samples were centrifuged at 3000-7000 x g at 4 °C for 30 seconds. The supernatants (unbound fractions) were removed. The beads were washed with 1X Buffer D + 0.2% Triton-X 100 (with protease inhibitors and N-ethylmaleimide) and then centrifuged at 7000 x g at 4 °C for 30 seconds. The supernatants were removed, and the washes were repeated three more times with 1X Buffer D + 0.2% Triton-X 100 (with protease inhibitors and N-ethylmaleimide) and three more times with 1X Buffer D + 0.5% Triton-X 100 (with protease inhibitors and N-ethylmaleimide). To elute bound proteins from the beads, the beads were re-suspended in 2X SDS lysis buffer and boiled for 10 minutes. The samples were centrifuged at 7000 x g at RT for 30 seconds, and the supernatants (bound fractions) were stored at -20°C.

2.8 Running SDS-PAGE Gels, Coomassie/Silver Staining, and Western Blotting

Proteins were resolved on 15%, 12.5% or 7.5% SDS-PAGE gels (indicated). For visualization of all resolved proteins in a gel, gels were stained in Coomassie Brilliant Blue

solution (0.025 % Coomassie Brilliant Blue Reagent, 40% methanol, 7% glacial acetic acid) until the desired band intensities were seen, and then de-stained in Destain solution (5% methanol, 7% glacial acetic acid) until background staining was removed. For a more sensitive detection of proteins, gels were silver-stained using Pierce Silver Stain Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

To perform Western blots, proteins from the gels were transferred to Polyvinylidene Fluoride (PVDF) membranes (a semi-dry transfer apparatus was used for 15% gels and a wet transfer apparatus was used for 12.5% and 7.5% gels). Membranes to be blotted with Avidin – horseradish peroxidase (AVI-HRP) (Sigma, A3151) were blocked in Tris Buffered Saline with Tween (TBS-T) + 4% bovine serum albumin (BSA) at RT for 1 hour or overnight at 4°C. After the membranes were blocked in BSA, they were incubated with AVI-HRP in TBS-T + 4% BSA at RT for 1 hour or overnight at 4°C. The membranes were then washed twice with TBS-T (15 minutes per wash), soaked in the appropriate enhanced chemiluminescence (ECL) substrate for 5 minutes, and developed using autoradiography film. Membranes to be blotted in all other primary antibodies were blocked in Tris Buffered Saline (TBS) + 5% milk at RT for 1 hour or overnight at 4°C. After blocking, the membranes were incubated with the appropriate primary antibody in TBS-T + 2% milk at RT for 1 hour or overnight at 4°C. The following primary antibodies were used in this study: monoclonal anti-Flag M2 (Sigma, F1365), anti-Flag (polyclonal) (Sigma, F7425), anti-phospho-H2A.X (Ser139) (Millipore, 07-164), and anti-HA.11 epitope tag (monoclonal) (Covance, MMS-101R). After incubation with primary antibodies, the membranes were washed twice with TBS-T (15 minutes per wash) and incubated with the appropriate secondary antibody in TBS-T + 2% milk at RT for 1 hour or overnight at 4°C. The following secondary antibodies were used in this study: goat anti-mouse IgG-HRP (Santa Cruz

Biotechnology, sc-2005) and goat anti-rabbit IgG-Fc fragment HRP conjugated (Bethyl, A120-111P). After incubation with secondary antibodies, the membranes were washed twice with TBS-T (15 minutes per wash), soaked in the appropriate ECL substrate for 5 minutes, and developed using autoradiography film.

CHAPTER 3: RESULTS

3.1 Identifying Proteins that Interact with Ubiquitylated H2A.X – Containing Nucleosomes in a DNA Damage-Dependent Manner

3.1.1 Characterizing the Ubiquitylation, Biotinylation Activity, and Nucleosomal Incorporation of H2A.X-BirA

In an effort to identify proteins that interact specifically with ubiquitylated H2A.X – containing nucleosomes, we began to develop a system that would tag ubiquitylated H2A.X histones with biotin *in vivo* **(Figure 9B)**. This system would involve the co-expression of an H2A.X-BirA fusion protein with Avi-tagged ubiquitin. If H2A.X-BirA becomes modified with Avi-tagged ubiquitin, BirA should be able to biotinylate the Avi-tagged ubiquitin due to their close proximity. Once the ubiquitylated H2A.X-BirA – containing nucleosomes are tagged with biotin, they can be purified with streptavidin-coupled beads, and interacting proteins could then be identified using mass spectrometry. In order to identify proteins that interact with ubiquitylated H2A.X-BirA – containing nucleosomes in a DNA damage – dependent manner, ubiquitylated H2A.X-BirA – containing nucleosomes would be purified from DNA damaged cells and non-DNA damaged cells. A comparison of interacting proteins between the two conditions would indicate which proteins are DNA-damage specific.

To begin, we designed the H2A.X-BirA fusion protein to contain an artificial Flag-tag between the H2A.X and BirA segments. The Flag-tag is comprised of a unique amino acid sequence (DYKDDDDK) that is not present within any endogenous mammalian proteins. Therefore, this tag would allow for easy detection of the protein with anti-flag antibodies on

Western blots, and purification of the protein by immunoprecipitation with anti-Flag antibodies. The H2A.X and Flag-tag segments are connected by a short amino acid linker (GGGS), and the Flag-tag and BirA segments are connected by a longer amino acid linker (GGGSGGGGT). The composition of each linker was designed to give BirA the maximum flexibility to 'flip' over and reach H2A.X.

Once the H2A.X-Flag-BirA construct was made, we tested if H2A.X-Flag-BirA (~52 kDa) could become biotinylated if it was ubiquitylated with Avi-tagged ubiquitin (~11 kDa). H2A.X-Flag-BirA was either singly expressed or co-expressed with Avi-tagged ubiquitin in mammalian 293T cells (human embryonic kidney cells). It wasn't necessary for us to induce DNA damage in these cells for H2A.X-Flag-BirA to become ubiquitylated, since H2A.X is known to have a basal level of monoubiquitylation on K118/119 at sites of transcriptional repression (Mattiroli et al., 2012). Nuclear lysates were prepared and subjected to Western blot analysis with anti-Flag antibodies and Avidin - horseradish peroxidase (AVI-HRP, which detects biotinylated substrates) **(Figure 10)**. As expected, the results showed that H2A.X-Flag-BirA could be biotinylated if it was ubiquitylated with Avi-tagged ubiquitin: when nuclear lysates were blotted with AVI-HRP, a single band around 63 kDa was observed, which is the approximate size of monoubiquitylated H2A.X-Flag-BirA with Avi-tagged ubiquitin. No such band was visible when H2A.X-Flag-BirA was singly expressed, indicating that biotinylation of H2A.X-Flag-BirA can only occur when it is modified with Avi-tagged ubiquitin. It was also noticed that only a fraction of H2A.X-Flag-BirA became ubiquitylated with Avi-tagged ubiquitin, as the majority of it still became ubiquitylated with endogenous ubiquitin. This finding was observed when nuclear lysates were blotted with anti-Flag antibodies: above the H2A.X-Flag-BirA band is a shifted band representing the monoubiquitylated form with

endogenous ubiquitin, and slightly above that band is a weaker band representing the monoubiquitylated form with Avi-tagged ubiqutin.

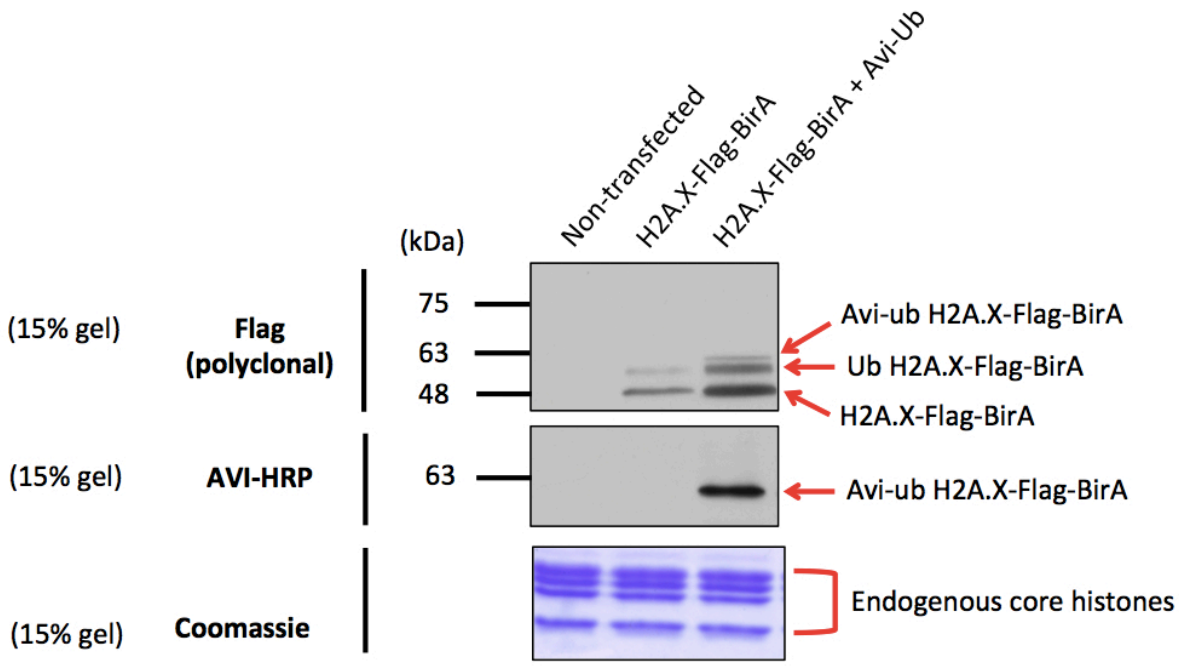


FIGURE 10. Determining if H2A.X-Flag-BirA is able to become biotinylated if it is ubiquitylated with Avi-tagged ubiquitin. H2A.X-Flag-BirA was either singly expressed or co-expressed with Avi-tagged ubiquitin in 293T cells. One cell sample was left untransfected. Nuclear lysates were prepared, and proteins were resolved on SDS-PAGE gels and subjected to Western blot analysis with the indicated antibodies. AVI-HRP was used to detect biotinylated substrates. Below the Western blots is a coomassie-stained gel showing the relative levels of the endogenous core histones present in the nuclear lysates, which acted as loading controls. Ub = ubiquitin/ubiquitylated. n= >3.

To verify that K118/119 was the site of basal monoubiquitylation observed, a plasmid expressing an H2A.X-K118/119R-Flag-BirA mutant was constructed. By changing the lysines to arginines, ubiquitylation can no longer occur at the 118/119 site. The wild-type (WT) and mutant H2A.X-Flag-BirA proteins were individually expressed in 293T cells, and their

ubiquitylation levels were assessed through Western blot analysis with nuclear lysates **(Figure 11)**. While monoubiquitylation was present on WT H2A.X-Flag-BirA, it was evident that the K118/119R mutation on H2A.X abolished its ubiquitylation, verifying that K118/119 is indeed the site of basal monoubiquitylation.

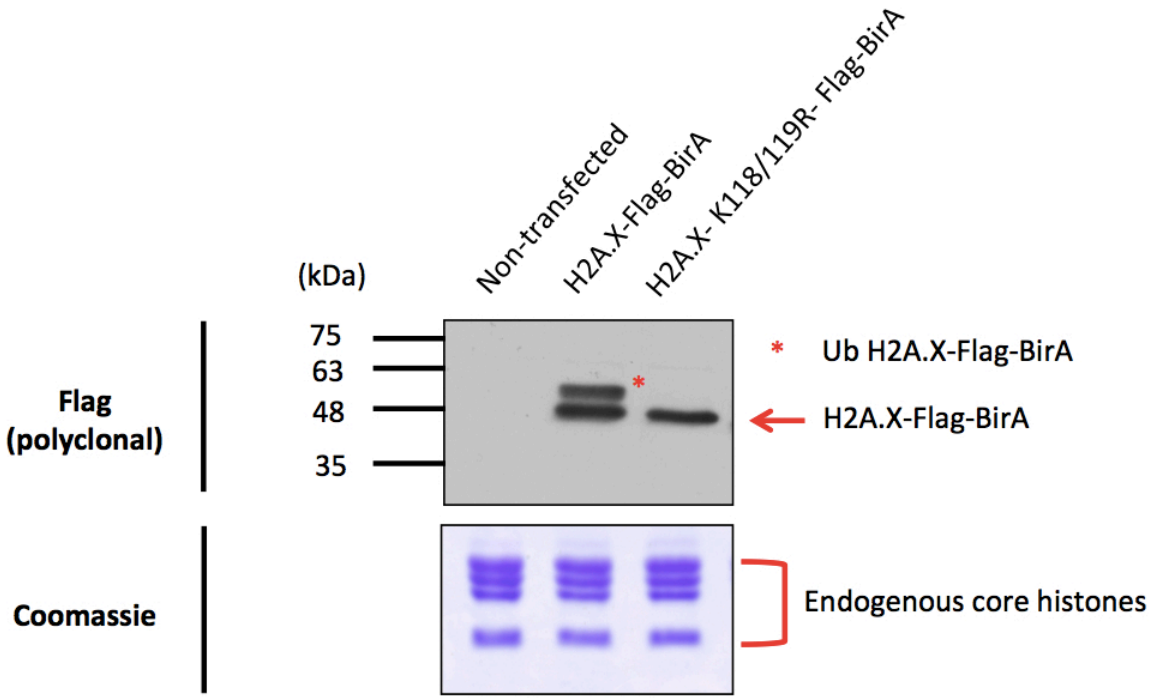
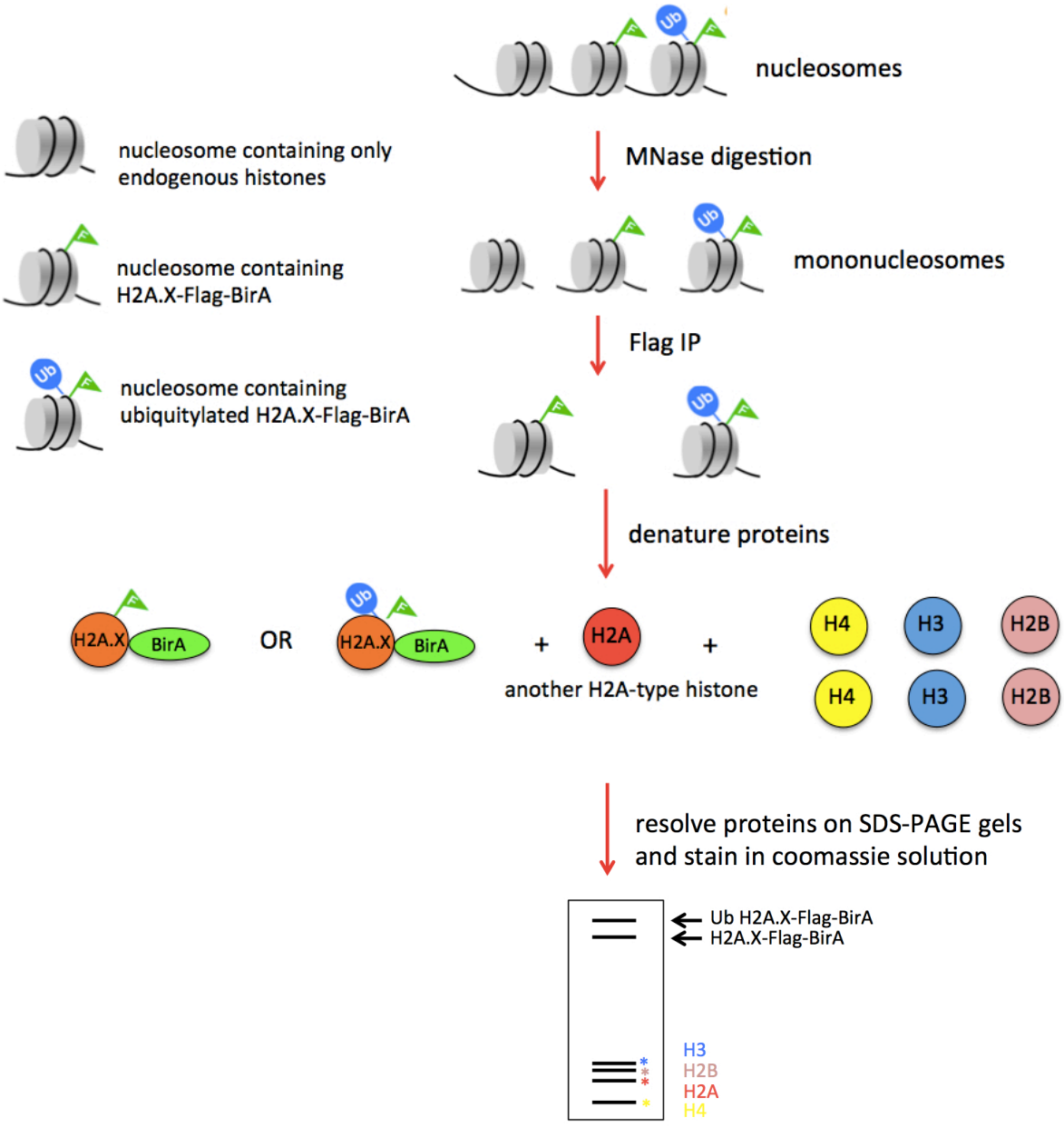


FIGURE 11. Verifying that K118/119 is the basal site of monoubiquitylation on H2A.X-Flag-BirA. H2A.X-Flag-BirA and H2A.X-K118/119R-Flag-BirA were individually expressed in 293T cells. One cell sample was left untransfected. Nuclear lysates were prepared, and proteins were resolved on an SDS-PAGE gel and subjected to Western blot analysis with anti-Flag antibodies. Below the Western blot is a coomassie-stained gel showing the relative levels of the endogenous core histones present in the nuclear lysates, which acted as loading controls. Ub = ubiquitin/ubiquitylated. n = >3.

To confirm H2A.X-Flag-BirA is properly incorporated into nucleosomes, we purified mononucleosomes and tested for stoichiometrical co-purification of other core histones with the fusion H2A.X. H2A.X-Flag-BirA was expressed in 293T cells, nuclei were isolated, and

chromatin was digested with micrococcal nuclease (MNase) to yield mononucleosomes **(Rationale 1)**. The lysates containing mononucleosomes and other nuclear proteins were then subjected to immunoprecipitation with anti-Flag antibodies to isolate H2A.X-Flag-BirA and potential protein interactors. To visualize all proteins that were co-purified with H2A.X-Flag-BirA, the immunoprecipitated proteins (referred to as 'bound' fraction) were denatured, resolved on an SDS-PAGE gel, and subsequently stained in coomassie brilliant blue solution **(Figure 12)**. The results showed that H2A.X-Flag-BirA is stably incorporated into nucleosomes, as the other core histones (H3, H2B, and H4) were co-purified with it in equimolar stoichiometry. Very low levels of endogenous H2A histones were also co-purified, indicating that that some nucleosomes containing H2A.X-Flag-BirA also contained an endogenous copy of an H2A-type histone.



H2A.X-Flag-BirA should be visible,
as well as the other core histones in equimolar stoichiometry

RATIONALE 1. Schematic of the method used to determine if H2A.X-Flag-BirA is incorporated into nucleosomes. To determine if H2A.X-Flag-BirA is incorporated into nucleosomes, we would have to purify H2A.X-Flag-BirA and see if the endogenous core histones (H2B, H3, H4 and H2A) would be co-purified with it. To do this, H2A.X-Flag-BirA would be expressed in cells, and then chromatin would be digested with MNase to obtain mononucleosomes. Mononucleosomes and other nuclear proteins would then be subjected to immunoprecipitation with anti-Flag antibodies (Flag IP) to isolate H2A.X-Flag-BirA and its ubiquitylated forms. Immunoprecipitated proteins would then be denatured, resolved on an SDS-PAGE gel and subsequently stained in coomassie solution for visualization. If H2A.X-Flag-BirA is incorporated into nucleosomes, we would expect the endogenous core histones to co-purify with it, and therefore be detected on the gel in equal amounts to H2A.X-Flag-BirA. Some components of this figure were made by Peter Cheung.

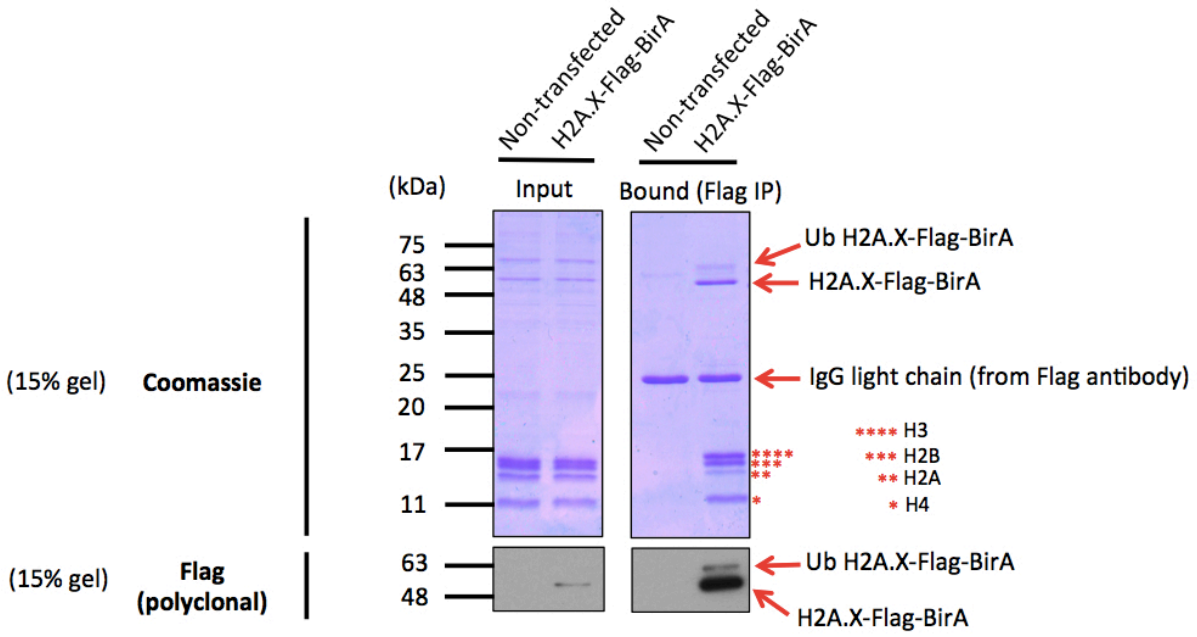


FIGURE 12. Determining if H2A.X-Flag-BirA is incorporated into nucleosomes. H2A.X-Flag-BirA was expressed in 293T cells. One cell sample was left untransfected. Chromatin from both samples was digested with MNase, and then mononucleosomes and other nuclear proteins were subjected to immunoprecipitation with anti-Flag antibodies. Immunoprecipitated proteins (bound fraction) and input material (proteins that were not subjected to immunoprecipitation) were resolved on an SDS-PAGE gel and stained in coomassie solution for visualization of all proteins. Immunoprecipiated proteins and input material were also subjected to Western blot analysis with anti-Flag antibodies. 0.3% of total input material and 71% of total immunoprecipitated material was loaded for each sample for the coomassie-stained gel, and 0.3% of total input material and 10% of total immunoprecipitated material was loaded for the Flag blot. Ub = ubiquitin/ubiquitylated. n = 1.

3.1.2 Testing and Optimizing Different DNA Damage – Inducing Agents

Our results have shown that H2A.X-Flag-BirA is monoubiquitylated at K118/119 under normal conditions. However, we are specifically interested with the DNA damage – induced ubiquitylation of H2A.X-Flag-BirA. Therefore, we began to test different DNA damage –

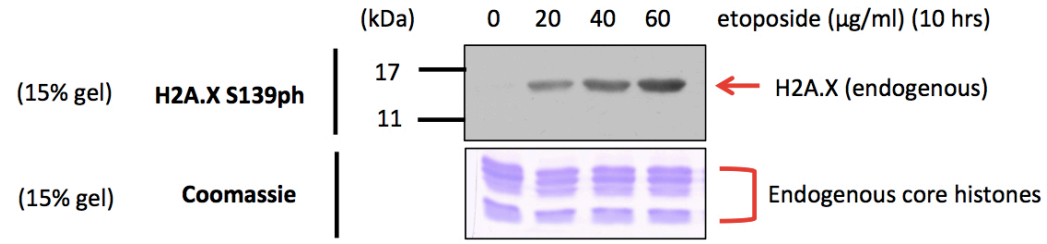
inducing agents to determine which one would be the most effective at inducing DNA damage (particularly, DSBs) in 293T cells.

We examined four different chemical DNA damage – inducing agents: etoposide, methyl methanesulfonate (MMS), doxorubicin, and neocarzinostatin (NCS). Etoposide and doxorubicin are both inhibitors of topoisomerase II enzymes: these enzymes cleave and re-ligate DNA to relieve torsional stress during DNA-related processes like replication and transcription. Etoposide temporarily binds to topoisomerase II enzymes after they have cleaved DNA strands to prevent re-ligation of the DNA. Etoposide results in the formation of transient single-strand breaks (SSBs) and transient DSBs, both of which be converted to stable DSBs upon replication or transcription (Muslimović et al., 2009). Doxorubicin inhibits topoisomerase II enzymes by inserting itself between adjacent base pairs, which prevents the ability of the enzymes to re-ligate the DNA, thus resulting in the formation of stable DSBs (Yang et al., 2015). MMS is an alkylating agent that damages bases by modifying them with methyl groups, which can stall replication fork progression, resulting in DSBs (Staszewski et al., 2008). NCS is an antibiotic that inserts itself into DNA and generates free radicals, which causes the formation of DSBs (Banin et al., 1998). Etoposide, doxorubicin and neocarzinostatin are used as drugs for the treatment of cancers, where their ability to induce DNA damage is used to kill cancer cells (Muslimović et al., 2009; Yang et al., 2015; Banin et al., 1998).

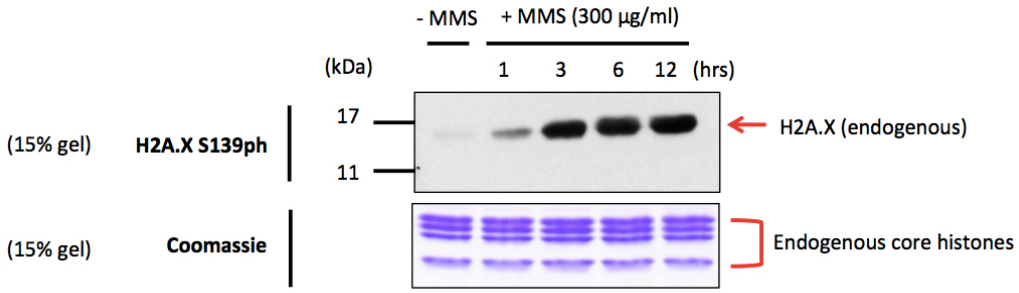
To optimize the ability of each drug to effectively induce DSBs, 293T cells were either treated with a drug at different concentrations for one fixed length of time, or treated with a drug at a specific concentration for different lengths of time. Levels of induced DSBs were evaluated by Western-blotting the nuclear lysates with an antibody for H2A.X S139 phosphorylation, which is a standard marker for DSBs. When cells were treated with etoposide for 10 hours at a

concentration of 20, 40 or 60 μg/ml, it was found that a concentration of 60 μg/ml induced the highest levels of H2A.X phosphorylation **(Figure 13A)**. When cells were treated with MMS at 300 μg/ml for 1, 3, 6 or 12 hours, levels of H2A.X phosphorylation appeared equally high from 3-12 hours **(Figure 13B)**. Upon treatment of cells with doxorubicin at 200 ng/ml for 24, 36 or 48 hours, H2A.X phosphorylation levels appeared similar from 24-48 hours, although a slight increase was observed after 48 hours **(Figure 13C)**. Unlike these three drugs, NCS was not effective at inducing H2A.X phosphorylation under the conditions we used: compared to an untreated cell sample, H2A.X phosphorylation levels were only slightly increased when cells were treated with NCS at 200 ng/ml for 15 minutes, 0.5, 1 or 3 hours **(Figure 13D)**. Overall, our results suggest the drugs would be most efficient (with respect to DSB induction and time) under the following conditions: etoposide at 60 μg/ml for 10 hours, MMS at 300 μg/ml for 3 hours, and doxorubicin at 200 ng/ml for 24 hours.

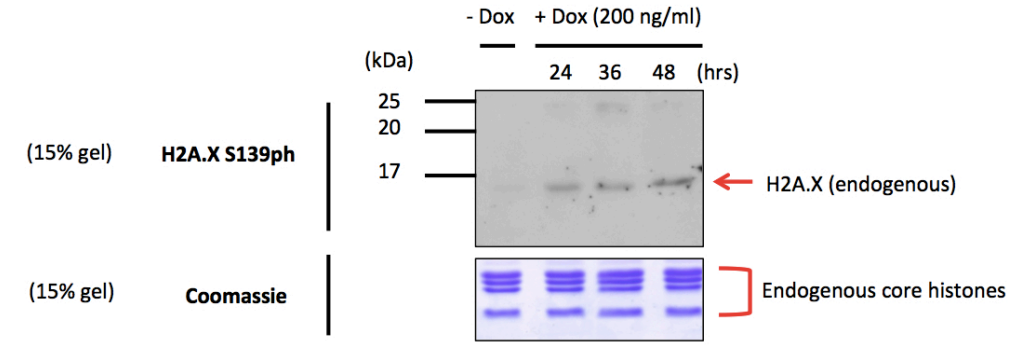
A


(kDa)
0
20
40
60
etoposide (μg/ml) (10 hrs)
(15% gel)
H2A.X S139ph
17
11
H2A.X (endogenous)
(15% gel)
Coomassie
Endogenous core histones

B


- MMS
+ MMS (300 μg/ml)
(kDa)
1
3
6
12
(hrs)
(15% gel)
H2A.X S139ph
17
11
H2A.X (endogenous)
(15% gel)
Coomassie
Endogenous core histones

C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+ Dox (200 ng/ml)
(kDa)
24
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25
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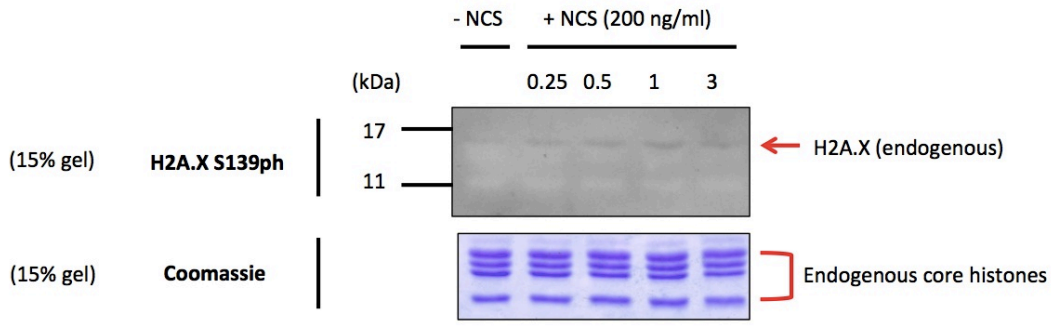
(15% gel)
H2A.X S139ph
H2A.X (endogenous)
(15% gel)
Coomassie
Endogenous core histones

D


- NCS
+ NCS (200 ng/ml)
(kDa)
0.25
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3
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(15% gel)
H2A.X S139ph
H2A.X (endogenous)
(15% gel)
Coomassie
Endogenous core histones

FIGURE 13. Testing and optimizing the efficiencies of different chemical DNA damage – inducing agents. 293T cells were left untreated, or were treated with **A)** etoposide at a concentration of 20, 40 or 60 μg/ml for 10 hours, **B)** MMS at a concentration of 300 μg/ml for 1, 3, 6 or 12 hours, **C)** doxorubicin (dox) at a concentration of 200 ng/ml for 24, 36 or 48 hours, or **D)** NCS at a concentration of 200 ng/ml for 0.25, 0.5, 1 or 3 hours. Nuclear lysates were prepared, and proteins were resolved on SDS-PAGE gels and subjected to Western blot analysis with anti-H2A.X S139 phosphorylation antibodies. H2A.X S139 phosphorylation was used to assess levels of DNA DSBs. Below the Western blots are coomassie-stained gels showing the relative levels of the endogenous core histones present in the nuclear lysates, which acted as loading controls. n = 1.

In addition to using chemicals, we also tried inducing DSBs using the CRISPR-Cas9 system. The CRISPR-Cas9 system has been adapted and developed to edit genomic DNA in mammalian cells. In this system, the nuclease Cas9 (originally from the bacterium *Streptomyces pyogenes*) is expressed alongside a guide RNA that has homology to a 20-nucleotide sequence found within the genome. As its name implies, the guide RNA guides Cas9 directly to the 20-nucleotide sequence in the genome, where it makes a DSB at a specific site within the sequence. If specific insertions, deletions or point mutations are desired, a repair template must be present. The repair template is usually a double-stranded DNA molecule containing the desired change with ends that are homologous to the regions flanking the 20-nucleotide sequence. The modified sequence on the repair template can then replace the original sequence in the genome through HR. If no repair template is provided, the DSBs usually become repaired through NHEJ. Since NHEJ can cause insertions and deletions, it can be useful for knocking out genes by generation of premature stop codons (Ran et al., 2013).

For our purpose, we took advantage of Cas9's ability to introduce DSBs in the genome at specific locations. To induce as many DSBs in the genome as possible, Cas9 should ideally be

targeted to highly repetitive sequences in the genome, and one such target might be Alu sequences since they are short, non-coding, and highly repetitive elements found throughout the human genome (over a million copies are in the human genome) (Häsler and Strub, 2006). As done by Xiao et al, we cloned a guide RNA that contains a homology sequence that would allow it to target 280,000 Alu sites to test whether this works to induce DSBs into the genome (McCaffrey et al., 2015).

The Cas9 nuclease (Flag-Cas9, 188 kDa) and Alu element guide RNA are expressed from the same plasmid (termed Cas9-Alu). We transfected different amounts of the Cas9-Alu plasmid into cells to determine which amount would be optimal. Along with the Cas9-Alu plasmid, we co-transfected plasmids expressing H2A.X-Flag-BirA and Avi-tagged ubiquitin. Ideally, the optimal amount of Cas9-Alu plasmid to transfect would be an amount that would induce the highest amount of DSBs without reducing the expression of H2A.X-Flag-BirA and Avi-tagged ubiquitin by much; it has been observed during co-transfections that the greater the amount of one plasmid transfected, the lower is the expression of the other co-transfected plasmids. The Cas9-Alu plasmid was co-transfected with plasmids expressing H2A.X-Flag-BirA and Avi-tagged ubiquitin in 293T cells at a ratio of 1:1:1, 2:1:1, 3:1:1, and 4:1:1, respectively. Nuclear lysates were prepared and subjected to Western blot analysis **(Figure 14)**. Results showed that a ratio of 1:1:1 induced the highest level of endogenous H2A.X phosphorylation while maintaining the highest level of H2A.X-Flag-BirA expression (seen on the Flag blot), therefore making it the optimal ratio to use.

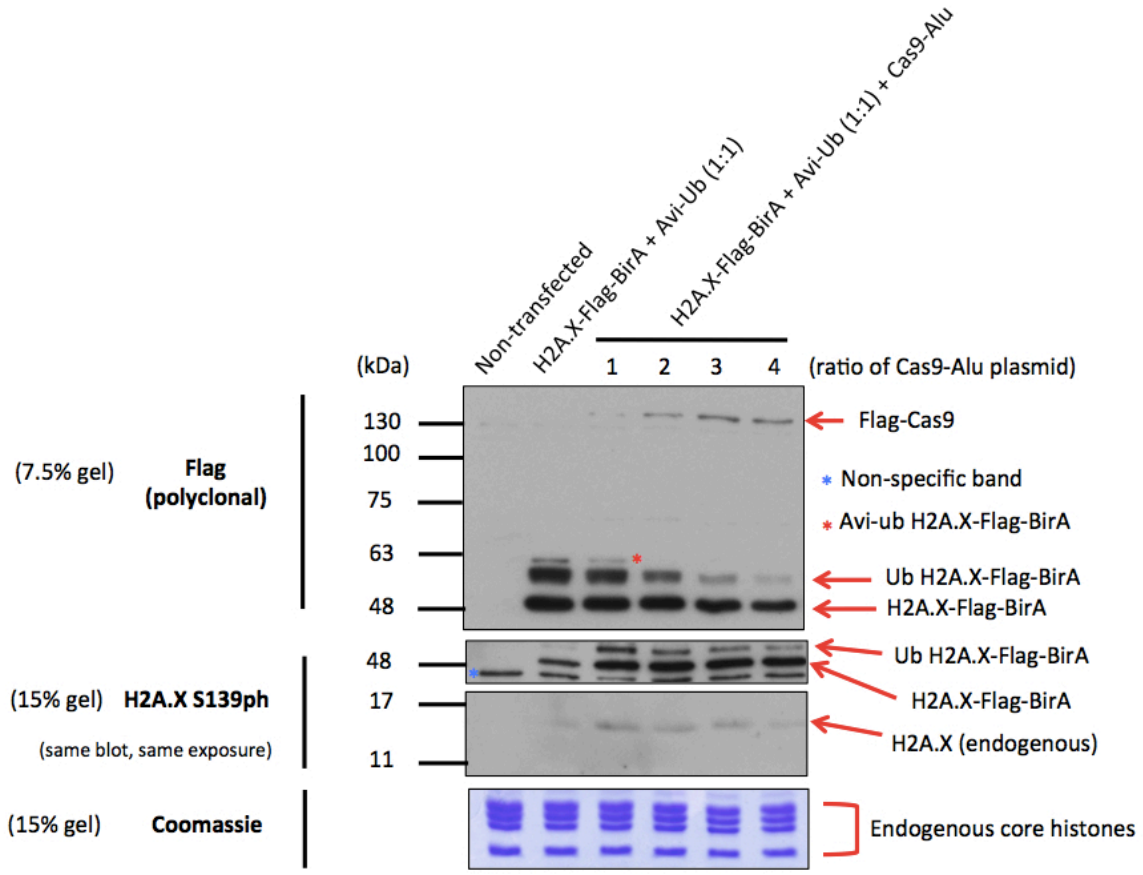


FIGURE 14. Testing and optimizing the ability of the CRISPR-Cas9 system to induce DNA damage. In 293T cells, H2A.X-Flag-BirA was either co-expressed with Avi-tagged ubiquitin at a 1:1 ratio, or co-expressed with both Avi-tagged ubiquitin and the Cas9-Alu plasmid at a ratio of 1:1:1, 1:1:2, 1:1:3, or 1:1:4, respectively. The Cas9-Alu plasmid was constructed to express the Cas9 endonuclease with a guide RNA targeted toward a human Alu element. One cell sample was left untransfected. Nuclear lysates were prepared, and proteins were resolved on SDS-PAGE gels and subjected to Western blot analysis with the indicated antibodies. H2A.X S139 phosphorylation was used to assess levels of DNA DSBs. Below the Western blots is a coomassie-stained gel showing the relative levels of the endogenous core histones present in the nuclear lysates, which acted as loading controls. Ub = ubiquitin/ubiquitylated. n = 1.

3.1.3 Collectively Evaluating the Efficiencies of Different DNA Damage – Inducing Agents and their Effects on the Ubiquitylation of H2A.X-Flag-BirA

After testing and optimizing different DNA damage – inducing agents, we compared their efficiencies in induction of DSBs to determine which one would be most suitable to use. We also examined the effects each agent had on the ubiquitylation of H2A.X-Flag-BirA. H2A.X-Flag-BirA and Avi-tagged ubiquitin were co-expressed in 293T cells, which were then left untreated, or were subjected to treatment with MMS, etoposide, or doxorubicin. The Cas9-Alu plasmid was also co-expressed in cells with H2A.X-Flag-BirA and Avi-tagged ubiquitin. Additionally, H2A.X-Flag-BirA was singly expressed in cells. Nuclear lysates were then prepared and subjected to Western blot analysis **(Figure 15)**. Among all the DNA damage – inducing agents, MMS was observed to be the most efficient at induction of DSBs, as it induced the highest levels of endogenous H2A.X phosphorylation. Etoposide, doxorubicin, and Cas9 appeared to be equally efficient at induction of DSBs, as they induced very similar levels of endogenous H2A.X phosphorylation.

The different DNA damage-inducing agents had different effects on the monoubiquitylation levels of H2A.X-Flag-BirA at K118/119. Compared to cells without induced DNA damage, MMS treatment and expression of the Cas9-Alu plasmid caused monoubiquitylation levels to decrease, while etoposide and doxorubicin treatments caused monoubiquitylation levels to increase. In particular, the Cas9-Alu plasmid resulted in the greatest decrease, and etoposide treatment resulted in the greatest increase. Changes in these monoubiquitylation levels were seen when the nuclear lysates were blotted with anti-Flag antibodies and AVI-HRP, although they are more evident on the AVI-HRP blot (at a low exposure).

Smears of shifted bands ranging from around 63-130 kDa were detected in samples treated with MMS, etoposide and doxorubicin (on the Flag blot at high exposure). These shifted bands are putatively polyubiquitylated forms of H2A.X-Flag-BirA. These increases in polyubiquitylated forms of H2A.X-Flag-BirA were expected since H2A.X histones have been reported to become polyubiquitylated in response to DSBs (polyubiquitin chains are formed upon the initial monoubiquitylation mark at K13/15) (Mattiroli et al., 2012). However, these polyubiquitylated forms were much less abundant than the monoubiquitylated form. Like the monoubiquitylated form, we would expect these polyubiquitylated forms of H2A.X-Flag-BirA to become biotinylated if they were modified with Avi-tagged ubiquitin. Therefore, we would expect to see similar banding patterns for these polyubiquitylated forms on the AVI-HRP blot. At a high exposure of the AVI-HRP blot, smears of bands between 63-130 kDa are seen in the MMS, etoposide and doxorubicin samples. However, the banding patterns seen on the AVI-HRP blot do not match the banding patterns seen on the Flag blot: the bands themselves appear to be different, and the general intensities of the bands are different (these bands are depicted within red boxes on the Flag and AVI-HRP blots). For example, on the AVI-HRP blot, the smear of bands was evidently much higher in intensity for the MMS sample compared to the etoposide and doxorubicin samples: this difference in intensity was not seen on the Flag blot. Therefore, these findings suggest that many of the bands seen on the AVI-HRP blot are not polyubiquitylated forms of H2A.X-Flag-BirA, but are other proteins that become ubiquitylated with Avi-tagged ubiquitin in response to DNA damage. This suggests that H2A.X-Flag-BirA is not only able to biotinylate ubiquitylated forms of itself, but also able to biotinylate other proteins ubiquitylated with Avi-tagged ubiquitin that are in close enough proximity (although to a much lesser extent than monoubiquitylated H2A.X-Flag-BirA). Because there is an increase in

the ubiquitylation of these proteins in response to DNA damage, their ubiquitylation may have a significant role in the repair of DSBs since the DNA damage response pathway is known to activate multiple ubiquitin-ligase enzymes. Therefore, this additional property of H2A.X-Flag-BirA may be advantageous, as we would have the potential of isolating these ubiquitylated proteins with streptavidin-coupled beads and determining their identities using mass spectrometry. As for the polyubiquitylated forms of H2A.X-Flag-BirA, it is unclear if any of them are able to become biotinylated, since it was difficult to detect them among the smears of other protein bands seen on the AVI-HRP blot. It is also important to note that on the AVI-HRP blot at high exposure, some bands were seen when H2A.X-Flag-BirA was expressed without Avi-tagged ubiquitin, signifying that H2A.X-Flag-BirA is able to biotinylate some proteins that are not modified with Avi-tagged ubiquitin. However, levels of those non-specific biotinylation are very low, and therefore shouldn't be of concern.

Overall, after examining the effects of each DNA damage – inducing agent, we decided to continue using MMS and etoposide for subsequent experiments. MMS induced the highest levels of endogenous H2A.X phosphorylation, caused increases in polyubiquitylated H2A.X-Flag-BirA, and resulted in high increases in the ubiquitylation of other proteins. Etoposide wasn't as efficient at increasing H2A.X phosphorylation levels or ubiquitylation of the other proteins, but it is interesting that it induced higher levels of monoubiquitylted H2A.X-Flag-BirA opposed to the decrease seen with MMS. Therefore, it would be interesting to compare proteins that interact with ubiquitylated H2A.X-Flag-BirA- containing nucleosomes in response to two different DNA – damage inducers.

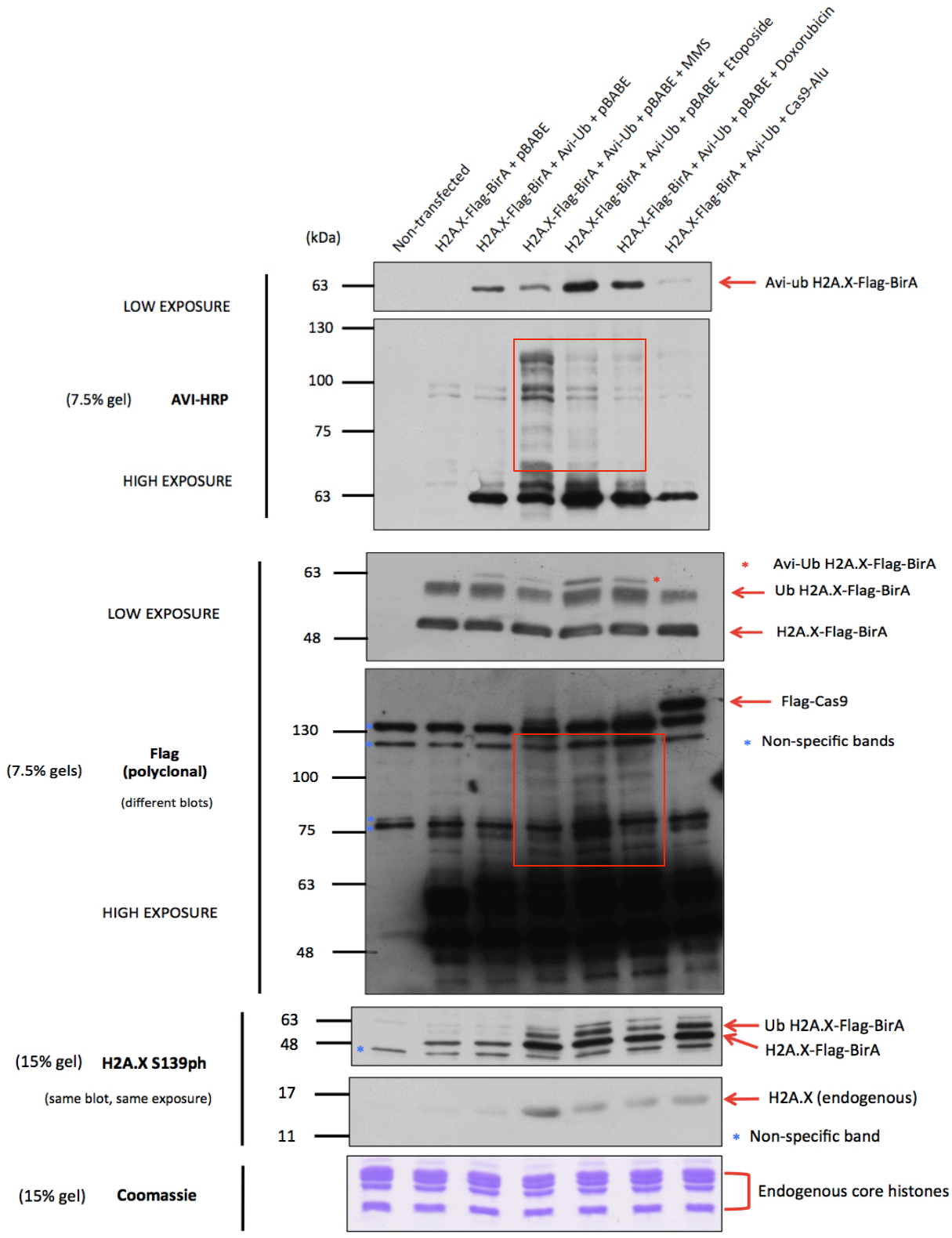

Non-transfected
H2A.X-Flag-BirA + pBABE
H2A.X-Flag-BirA + Avi-Ub + pBABE
H2A.X-Flag-BirA + Avi-Ub + pBABE + MMS
H2A.X-Flag-BirA + Avi-Ub + pBABE + Etoposide
H2A.X-Flag-BirA + Avi-Ub + pBABE + Doxorubicin
H2A.X-Flag-BirA + Avi-Ub + Cas9-Alu
(kDa)
LOW EXPOSURE
(7.5% gel) AVI-HRP
HIGH EXPOSURE
63
130
100
75
63
Avi-ub H2A.X-Flag-BirA
LOW EXPOSURE
(7.5% gels) Flag (polyclonal)
(different blots)
HIGH EXPOSURE
63
48
130
100
75
63
48
Avi-Ub H2A.X-Flag-BirA
Ub H2A.X-Flag-BirA
H2A.X-Flag-BirA
Flag-Cas9
Non-specific bands
(15% gel) H2A.X S139ph
(same blot, same exposure)
63
48
17
11
Ub H2A.X-Flag-BirA
H2A.X-Flag-BirA
H2A.X (endogenous)
Non-specific band
(15% gel) Coomassie
Endogenous core histones

FIGURE 15. Collectively evaluating the efficiencies of different DNA damage – inducing agents and their effects on the ubiquitylation of H2A.X-Flag-BirA. H2A.X-Flag-BirA was co-expressed with Avi-tagged ubiquitin in 293T cells, and treated with etoposide (60 μg/ml for 10 hours), MMS (300 μg/ml for 3 hours), or doxorubicin (200 ng/ml for 24 hours). H2A.X-Flag-BirA was also co-expressed with Avi-tagged ubiquitin and the Cas9-Alu plasmid in 293T cells (at a ratio of 1:1:1). The Cas9-Alu plasmid was constructed to express the Cas9 endonuclease with a guide RNA targeted toward a human Alu element. H2A.X-Flag-BirA was also singly expressed in 293T cells. In samples where the Cas9-Alu plasmid wasn't expressed, an empty pBABE vector was expressed to normalize the total amount of transfected plasmids across the samples. One cell sample was left untransfected. Nuclear lysates were prepared, and proteins were resolved on SDS-PAGE gels and subjected to Western blot analysis with the indicated antibodies. AVI-HRP was used to detect biotinylated substrates, and H2A.X S139 phosphorylation was used to assess levels of DNA DSBs. Below the Western blots is a coomassie-stained gel showing the relative levels of the endogenous core histones present in the nuclear lysates, which acted as loading controls. Note: Red boxes were drawn on the high exposures of the Flag and AVI-HRP blots to highlight differences in the banding patterns seen. Ub = ubiquitin/ubiquitylated. n = 1.

3.1.4 Examining the Effects of DNA Damage on the Ubiquitylation of H2A.X-Flag-BirA and the Ubiquitylation of the Other Biotinylated Proteins in Different Cell Lines

When we began our experiments, we initially chose to use 293T cells because they proliferate at higher rates and have the potential to express exogenous proteins at higher levels compared to most cell lines. These characteristics are due to the stable expression of the Simian Virus 40 (SV40) large T antigen in these cells: 293T cells were derived from 293 cells that were stably transfected with a plasmid encoding the SV40 large T antigen (DuBridge et al., 1987). The T antigen can increase the expression of proteins that are encoded by plasmids that contain an SV40 origin of replication and SV40 enhancers: it binds to the origin of replication to increase plasmid copy number (Nakanishi et al., 2008) and binds to the enhancers to enhance transcription of the gene (Kelly and Wildeman, 1991). The T antigen can also bind to other

proteins in the cell and affect their normal functioning. For example, it can bind to Retinoblastoma (Rb) proteins and prevent them from regulating the cell cycle, which causes the cells to enter S-phase more often, leading to higher proliferation rates. The T antigen can also bind to the tumour suppressor p53 and inhibit its activities (Ahuja et al., 2005). One function of p53 is to induce cell cycle arrest (or apoptosis in extreme conditions) when DSBs occur: the ATM kinase (also responsible for phosphorylation of H2A.X) phosphorylates Chk2, which in turn phosphorylates p53 to activate it. p53 induces transcription of genes involved in initiating cell cycle arrest to give the cells time to repair the break. It has also been reported that p53 plays roles in the regulation of HR and NHEJ (Menon and Povirk, 2014). Evidently, p53 has a connection to DSB signaling and repair.

Based on what is known about p53 from literature, it doesn't appear to participate in the early DSB signaling events surrounding H2A.X phosphorylation and ubiquitylation. Therefore, we didn't think that a loss in p53 activity would affect normal H2A.X ubiquitylation in response to DSBs. However, we still wanted to confirm if H2A.X ubiquitylation would be similar in cells with functional p53 versus cells with compromised p53. Additionally, we wanted to determine if an increase in the ubiquitylation of the other biotinylated proteins would still be seen during DNA damage if functional p53 were present. To answer these questions, H2A.X-Flag-BirA was co-expressed with Avi-tagged ubiquitin in 293T cells and 293 cells (which have normal p53 function due to the absence of the T antigen). Cells were treated with MMS or sterile water (the solvent MMS was dissolved in, used as a control). Nuclear lysates were then subjected to Western blot analysis **(Figure 16)**. Similar to 293T cells, MMS treatment caused a decrease in the monoubiquitylation of H2A.X-Flag-BirA in 293 cells (seen on the low exposures of the Flag and AVI-HRP blots). Additionally, ubiquitylation of some of the other biotinylated proteins

(above 75 kDa) were seen to increase with MMS treatment in both 293T and 293 cells (seen on the high exposure of the AVI-HRP blot). However, unlike 293T cells, polyubiquitylated forms of H2A.X-Flag-BirA could not be detected in 293 cells (at a high exposure of the Flag blot). This could have been a result of the lower expression of H2A.X-Flag-BirA in 293 cells compared to 293T cells. Despite this, the consistent drop in monoubiquitylated H2A.X-Flag-BirA and increase in ubiquitylation of other biotinylated proteins seen in both 293 and 293T cells give us enough confidence that the dynamics of H2A.X ubiquitylation and ubiquitylation of the other biotinylated proteins are not affected by the functional status of p53.

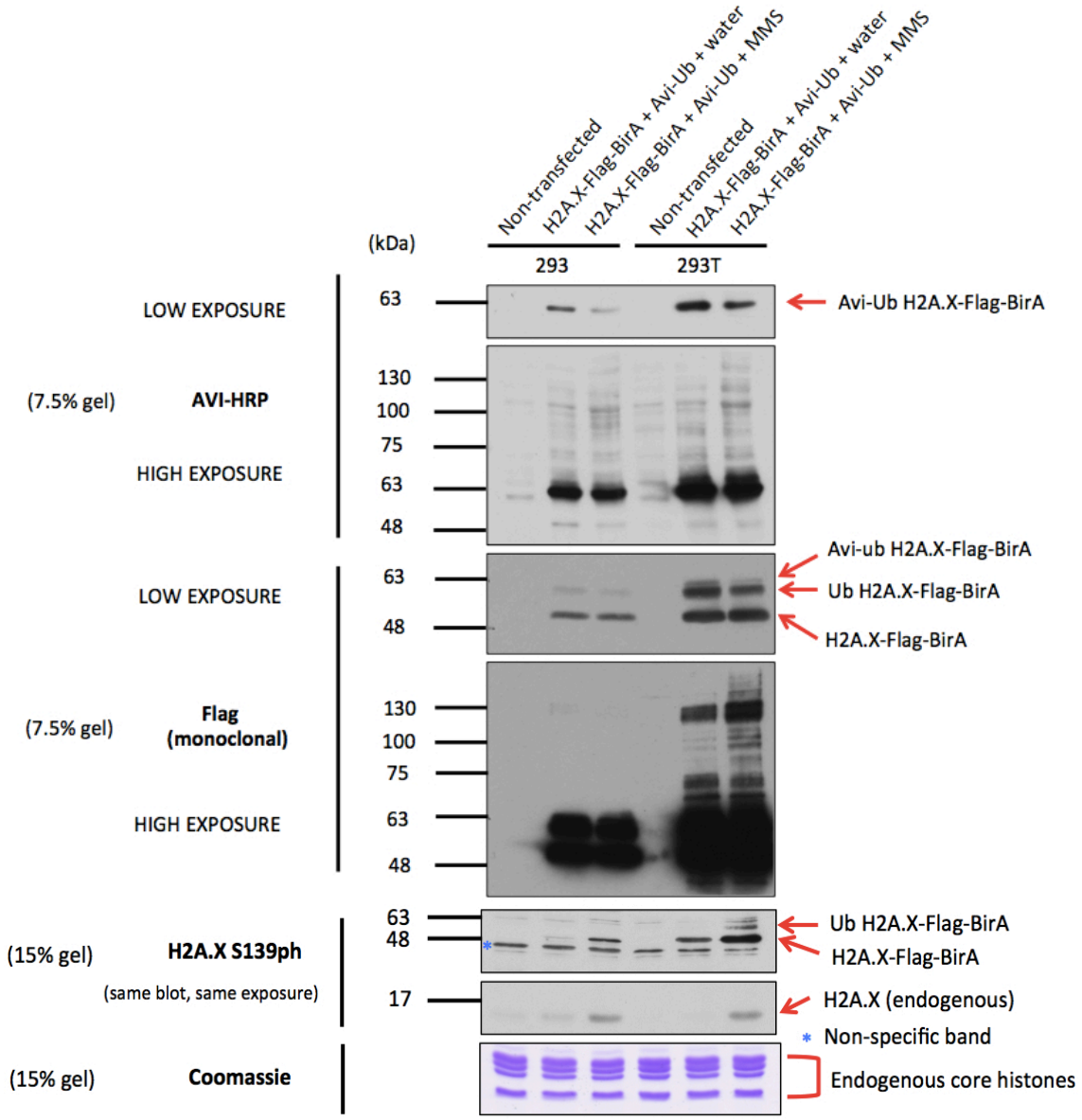


FIGURE 16. Comparing the effects of MMS treatment on the ubiquitylation of H2A.X-Flag-BirA and the ubiquitylation of the other biotinylated proteins between 293T and 293 cells. H2A.X-Flag-BirA was co-expressed with Avi-tagged ubiquitin in both 293T and 293 cells, which were either treated with MMS at 300 µg/ml for 3 hours, or sterile water for 3 hours (the solvent MMS was dissolved in, used as a control). For both 293T and 293 cells, one cell sample was left untransfected. Nuclear lysates were prepared, and proteins were resolved on SDS-PAGE gels and subjected to Western blot analysis with the indicated antibodies. AVI-HRP was used to detect biotinylated substrates, and H2A.X S139 phosphorylation was used to assess levels of DNA DSBs. Below the Western blots is a coomassie-stained gel showing the relative levels of the endogenous core histones present in the nuclear lysates, which acted as loading controls. Ub = ubiquitin/ubiquitylated. n = 2.

Apart from the 293 cell lineage, we also observed the effects DNA damage had on the ubiquitylation of H2A.X-Flag-BirA and the other biotinylated proteins in a different cell line. We tested U2OS cells (human bone cancer cells): although they do not proliferate as fast at 293T cells, they are easy to transfect, and express exogenous proteins well. H2A.X-Flag-BirA was co-expressed with Avi-tagged ubiquitin in 293T cells and U2OS cells, and treated with MMS or sterile water. Nuclear lysates were prepared and subjected to Western blot analysis **(Figure 17)**. As expected, the effects MMS had on U2OS cells were similar to the effects seen by MMS on 293T cells in previous experiments: there was a decrease in monoubiquitylated H2A.X-Flag-BirA (seen on the AVI-HRP blot and Flag blot at low exposure), an increase in polyubiquitylated forms of H2A.X-Flag-BirA (seen on the Flag blot at high exposure, between 75-100 kDa), and increases in the ubiquitylation of the other biotinylated proteins (seen on the AVI-HRP blot, above 63 kDa). Unexpectedly, however, polyubiquitylated H2A.X-Flag-BirA and the other ubiquitylated/biotinylated proteins were not detected in 293T cells in this particular experiment. Since these ubiquitylated substrates are normally easily detected, it is probable that they became degraded or were lost during nuclear lysis. Therefore, it is unlikely that these ubiquitylated substrates are actually more abundant in U2OS cells as they appeared on these blots.

After seeing how DNA damage had similar effects on all three cell lines, we decided to continue using 293T cells for all subsequent experiments, for the same reasons we initially began to use them: their fast growth rates and high expression of exogenous proteins.

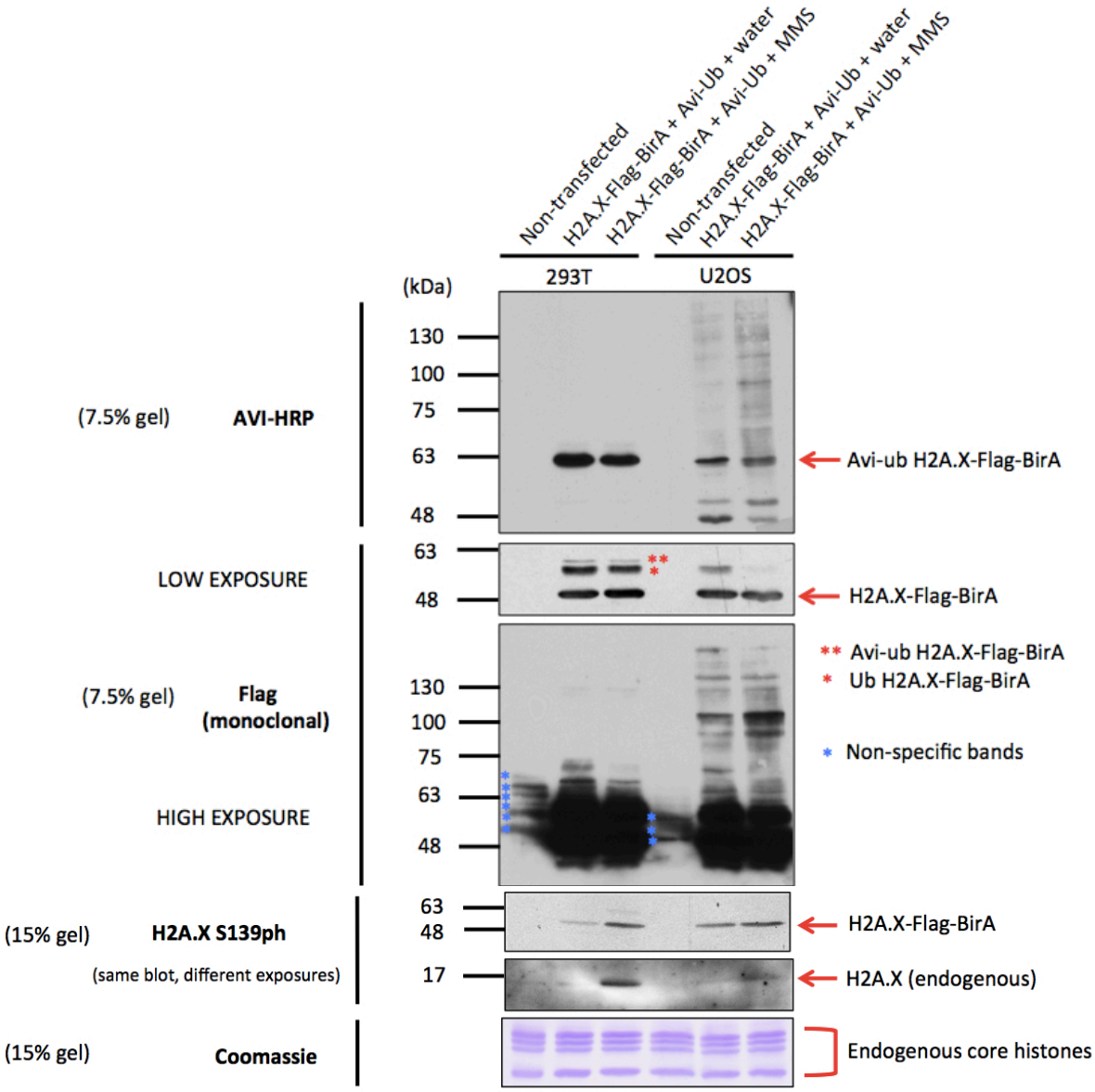


FIGURE 17. Comparing the effects of MMS treatment on the ubiquitylation of H2A.X-Flag-BirA and the ubiquitylation of the other biotinylated proteins between 293T and U20S cells. H2A.X-Flag-BirA was co-expressed with Avi-tagged ubiquitin in both 293T and U2OS cells, which were either treated with MMS at 300 µg/ml for 3 hours, or sterile water for 3 hours (the solvent MMS was dissolved in, used as a control). For both 293T and U2OS cells, one cell sample was left untransfected. Nuclear lysates were prepared, and proteins were resolved on SDS-PAGE gels and subjected to Western blot analysis with the indicated antibodies. AVI-HRP was used to detect biotinylated substrates, and H2A.X S139 phosphorylation was used to assess levels of DNA DSBs. Below the Western blots is a coomassie-stained gel showing the relative levels of the endogenous core histones present in the nuclear lysates, which acted as loading controls. Ub = ubiquitin/ubiquitylated. n = 1.

3.1.5 Determining if Polyubiquitylated Forms of H2A.X-Flag-BirA can Become Biotinylated

In the experiments described above, when H2A.X-Flag-BirA and Avi-tagged ubiquitin were co-expressed in cells subjected to MMS, etoposide or doxorubicin treatments, we saw an increase in the ubiquitylation of other biotinylated proteins: these proteins were detected as a smear of bands seen when the nuclear lysates were Western blotted with AVI-HRP **(Figures 15, 16 and 17)**. As mentioned previously, within these smears, it is difficult to distinguish polyubiquitylated forms of H2A.X-Flag-BirA from other proteins that become ubiquitylated. This makes it difficult to determine if any polyubiquitylated forms of H2A.X-Flag-BirA are able to become biotinylated. To address this issue, we separated H2A.X-Flag-BirA and all of its ubiquitylated forms from all the other nuclear proteins: this was done by subjecting nuclear lysates to immunoprecipitation with anti-Flag antibodies. All proteins in the nuclear lysates were denatured beforehand to abolish all protein-protein interactions: this was necessary to ensure that only H2A.X-Flag-BirA and its modified forms could be purified, and that no other proteins could become co-purified with them. By Western blotting the immunoprecipitated proteins with AVI-HRP, we would be able to determine if any of the polyubiquitylated forms of H2A.X-Flag-BirA become biotinylated.

H2A.X-Flag-BirA and Avi-tagged ubiquitin were co-expressed in 293T cells, which were then left untreated, or were treated with MMS or etoposide. Nuclear lysates were prepared, and then proteins were denatured and subjected to immunoprecipitation with anti-Flag antibodies. It was verified that protein-protein interactions were successfully disrupted, allowing only H2A.X-Flag-BirA to become isolated: this was confirmed by the absence of the core histones and other interacting proteins in the immunoprecipitated (bound) fractions, seen on a coomassie-stained gel

(Figure 18). The immunoprecipitated proteins were then subjected to Western blot analysis **(Figure 18)**. Based on the results obtained, it appears that polyubiquitylated forms of H2A.X-Flag-BirA are not able to become biotinylated; although polyubiquitylated forms of H2A.X-Flag-BirA were immunoprecipitated in all samples (seen on a high exposure of the Flag blot, between 63-75 kDa), they could not be detected by AVI-HRP. Only monoubiquitylated and di-ubiquitylated H2A.X-Flag-BirA were detected by AVI-HRP in the immunoprecipitated samples, suggesting that only these ubiquitylated forms can be biotinylated (presumably, the di-ubiquitylated form is monoubiquitylated at both K118/119 and K13/15). Therefore, in addition to the other ubiquitylated/biotinylated proteins, only these ubiquitylated forms of H2A.X-Flag-BirA have the potential of being purified with streptavidin-coupled beads.

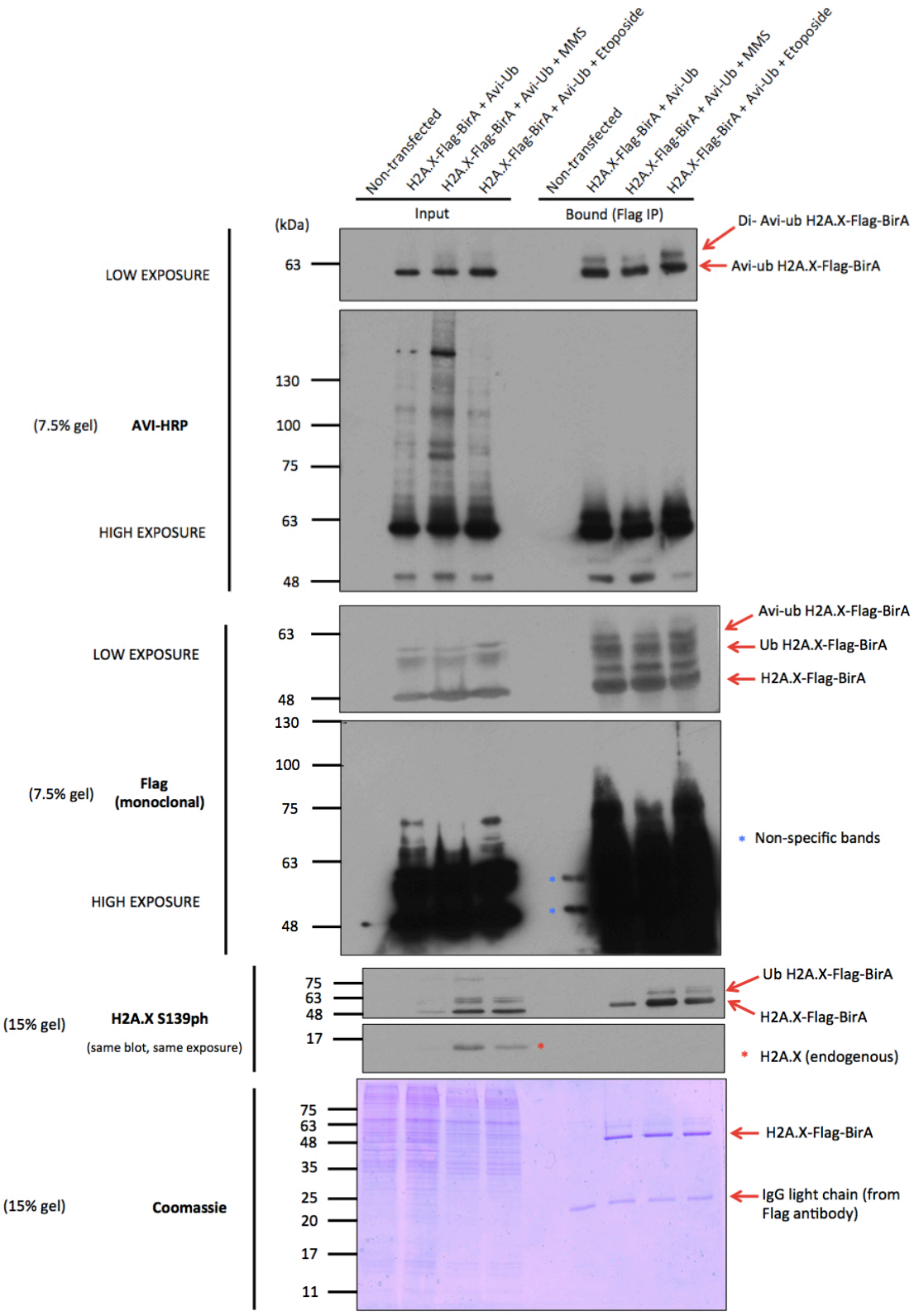

Non-transfected
H2A.X-Flag-BirA + Avi-Ub
H2A.X-Flag-BirA + Avi-Ub + MMS
H2A.X-Flag-BirA + Avi-Ub + Etoposide
Non-transfected
H2A.X-Flag-BirA + Avi-Ub
H2A.X-Flag-BirA + Avi-Ub + MMS
H2A.X-Flag-BirA + Avi-Ub + Etoposide
Input
Bound (Flag IP)
(kDa)
LOW EXPOSURE
63
Di- Avi-ub H2A.X-Flag-BirA
Avi-ub H2A.X-Flag-BirA
(7.5% gel)
AVI-HRP
130
100
75
63
48
HIGH EXPOSURE
LOW EXPOSURE
63
48
Avi-ub H2A.X-Flag-BirA
Ub H2A.X-Flag-BirA
H2A.X-Flag-BirA
(7.5% gel)
Flag
(monoclonal)
130
100
75
63
48
HIGH EXPOSURE
* Non-specific bands
(15% gel)
H2A.X S139ph
(same blot, same exposure)
75
63
48
17
Ub H2A.X-Flag-BirA
H2A.X-Flag-BirA
* H2A.X (endogenous)
(15% gel)
Coomassie
75
63
48
35
25
20
17
11
H2A.X-Flag-BirA
IgG light chain (from
Flag antibody)

FIGURE 18. Determining if polyubiquitylated forms of H2A.X-Flag-BirA can be biotinylated. H2A.X-Flag-BirA was co-expressed with Avi-tagged ubiquitin in 293T cells, which were either left untreated, or were treated with MMS at 300 μg/ml for 3 hours or etoposide at 60 μg/ml for 10 hours. One cell sample was left untransfected. Nuclear lysates were prepared, and proteins were denatured to abolish protein-protein interactions. Nuclear lysates were then subjected to immunoprecipitation with anti-Flag antibodies. Immunoprecipitated proteins (bound fraction) and input material (proteins that were not subjected to immunoprecipitation) were subjected to Western blot analysis with the indicated antibodies. AVI-HRP was used to detect biotinylated substrates, and H2A.X S139 phosphorylation was used to assess levels of DNA DSBs. Immunoprecipiated proteins and input material were also resolved on an SDS-PAGE gel and stained in coomassie solution for visualization of all proteins. The following percentages of total input material and total immunoprecipitated material were loaded for each sample: 0.4% input and 20% immunoprecipitated (for AVI-HRP blot), 0.8% input and 40% immunoprecipitated (for Flag blot), 0.3% input and 15% immunoprecipitated (for H2A.X S139ph blot) and 0.2% input and 10% immunoprecipitated (for the coomassie-stained gel). Ub = ubiquitin/ubiquitylated. n = 2.

3.1.6 Determining if the Other Ubiquitylated/Biotinylated Proteins are Associated with Nucleosomes

Theoretically, the enzymatic activity of BirA is proximity-dependent such that it should only be able to biotinylate Avi-tagged substrates in close proximity to itself. Therefore, the other ubiquitylated/biotinylated proteins should be associated with H2A.X in some way, either by direct or indirect interaction. Unfortunately, it is difficult to determine if these proteins are specifically associated with H2A.X-Flag-BirA, as there is no easy way of only being able to purify H2A.X-Flag-BirA and its protein interactors without also purifying the other core histones and their interactors. However, by immunoprecipitating H2A.X-Flag-BirA - containing nucleosomes, we could at least be able to determine if the ubiquitylated/biotinylated proteins are associated with nucleosomes. To do this, H2A.X-Flag-BirA and Avi-tagged ubiquitin were co-expressed in 293T cells and either treated with MMS or sterile water. Nuclear lysates were then

prepared in a manner that would allow protein-protein interactions to be preserved. The nuclear lysates were then subjected to immunoprecipitation with anti-Flag antibodies, and the immunoprecipitated proteins (bound fraction) were then subjected to Western blot analysis **(Figure 19)**. As expected, the ubiquitylated/biotinylated proteins were found to be associated with nucleosomes: proteins from 63-130 kDa were co-purified with H2A.X-Flag-BirA - containing nucleosomes, as seen on a high exposure of the AVI-HRP blot. It should be noted that chromatin wasn't digested with MNase in this experiment, so theoretically oligonucleosomes of different lengths were obtained instead of mononucleosomes after nuclear lysis. The protocol for obtaining mononucleosomes is long and many proteins become lost throughout the process, leaving only a small fraction of mononucleosomes and other nuclear proteins that are successfully extracted at the end. In comparison, the protocol for obtaining oligonucleosomes and other nuclear proteins is much shorter, and a larger fraction of proteins are extracted at the end. For this reason, isolation of H2A.X-Flag-BirA – containing nucleosomes in all subsequent experiments was done with oligonucleosomes as input material.

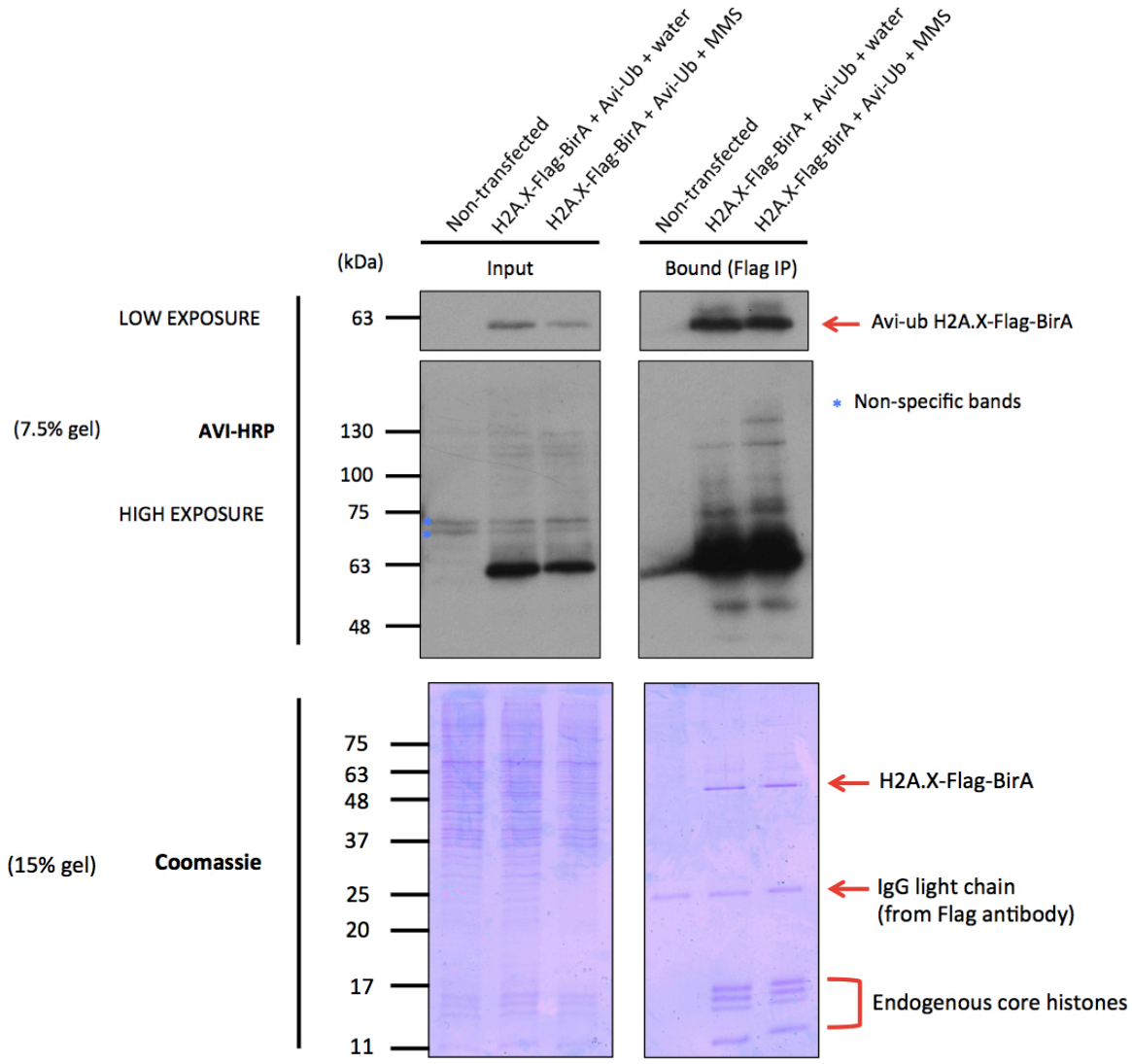


FIGURE 19. Determining if the other ubiquitylated/biotinylated proteins are associated with nucleosomes. H2A.X-Flag-BirA was co-expressed with Avi-tagged ubiquitin in 293T cells, which were either treated with MMS at 300 µg/ml for 3 hours, or sterile water for 3 hours (the solvent MMS was dissolved in, used as a control). One cell sample was left untransfected. Nuclear lysates were prepared in a manner that would preserve protein-protein interactions. Nuclear lysates were then subjected to immunoprecipitation with anti-Flag antibodies. Immunoprecipitated proteins (bound fraction) and input material (proteins that were not subjected to immunoprecipitation) were subjected to Western blot analysis with AVI-HRP, which was used to detect biotinylated substrates. Immunoprecipiated proteins and input material were also resolved on an SDS-PAGE gel and stained in coomassie solution for visualization of all proteins. 1% of total input material and 80% of total immunoprecipitated material was loaded for each sample for the AVI-HRP blot, and 0.2% of total input material and 10% of total immunoprecipitated material was loaded for the coomassie-stained gel. Ub = ubiquitin/ubiquitylated. n = 1.

3.1.7 Pulling Down Biotinylated Proteins Using Streptavidin-Coupled Beads

Throughout our previous experiments, we have seen that H2A.X-Flag-BirA is able to biotinylate mono and di-ubiquitylated forms of itself, as well as other DNA damage-related proteins when such substrates are modified with Avi-tagged ubiquitin. However, we had yet to test if these biotinylated substrates could effectively be pulled down using streptavidin-coupled beads. To do so, H2A.X-Flag-BirA was co-expressed with Avi-tagged ubiquitin in 293T cells, and subsequently treated with MMS. Nuclear lysates were prepared, and proteins were denatured to abolish protein-protein interactions. After the lysates were incubated with the beads, bound proteins were eluted and subjected to Western blot analysis **(Figure 20)**. As expected, monoubiquitylated and di-ubiquitylated H2A.X-Flag-BirA were effectively pulled down (seen on the Flag and AVI-HRP blots), with the other ubiquitylated/biotinylated proteins (seen as a smear of bands from 48-130 kDa on the AVI-HRP blot).

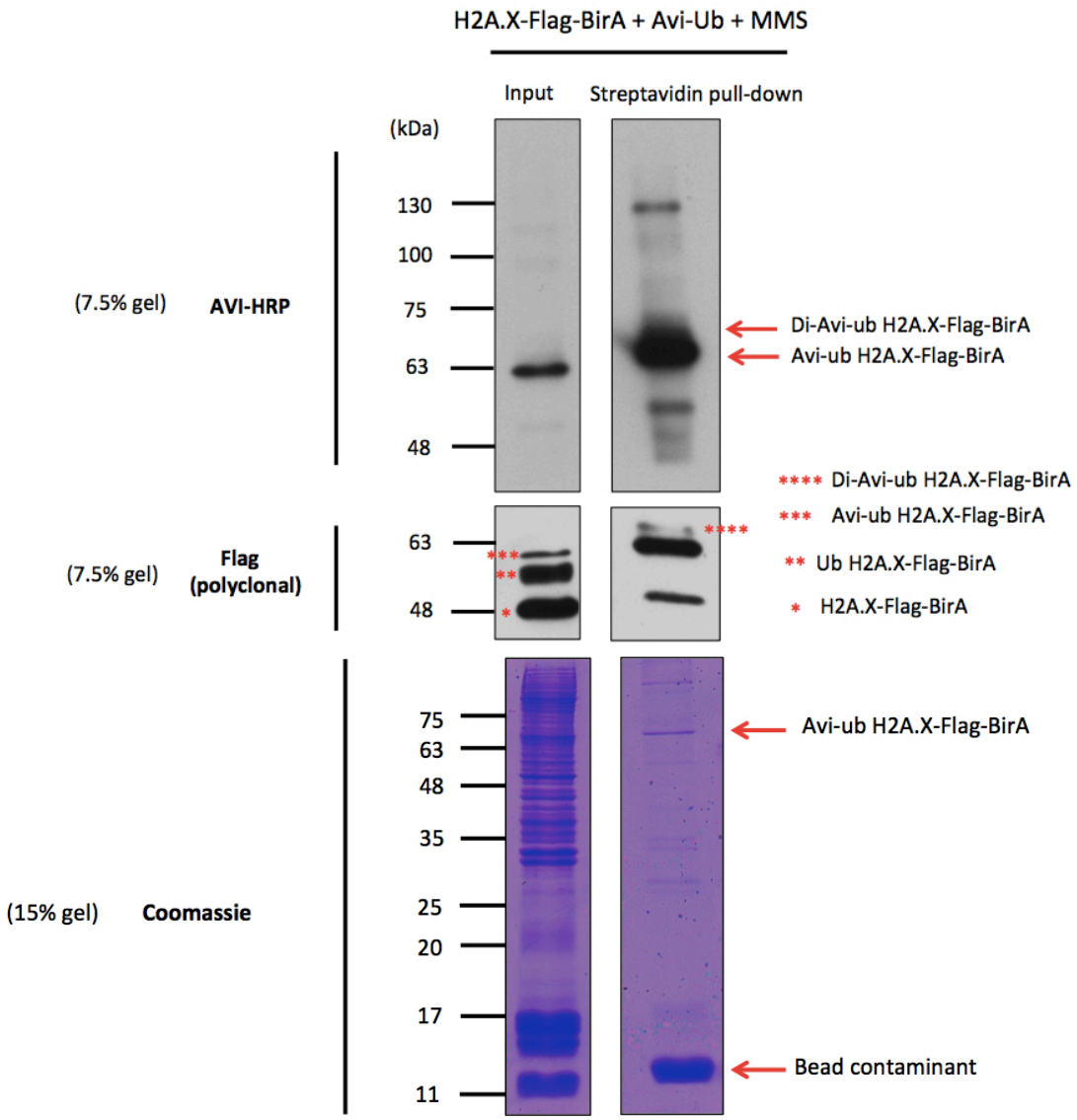


FIGURE 20. Testing if biotinylated proteins can be pulled down using streptavidin-coupled beads. H2A.X-Flag-BirA was co-expressed with Avi-tagged ubiquitin in 293T cells, and treated with MMS at 300 µg/ml for 3 hours. Nuclear lysates were prepared, and proteins were denatured to abolish protein-protein interactions. Nuclear lysates were then subjected to pull-downs with streptavidin-coupled beads to isolate biotinylated proteins. Pulled-down proteins and input material (proteins that were not subjected to pull-downs) were subjected to Western blot analysis with the indicated antibodies. AVI-HRP was used to detect biotinylated substrates. Pulled-down proteins and input material were also resolved on an SDS-PAGE gel and stained in coomassie solution for visualization of all proteins. 0.8% of total input material and 27% of total pull-down material was loaded for each sample. Ub = ubiquitin/ubiquitylated. n = 1.

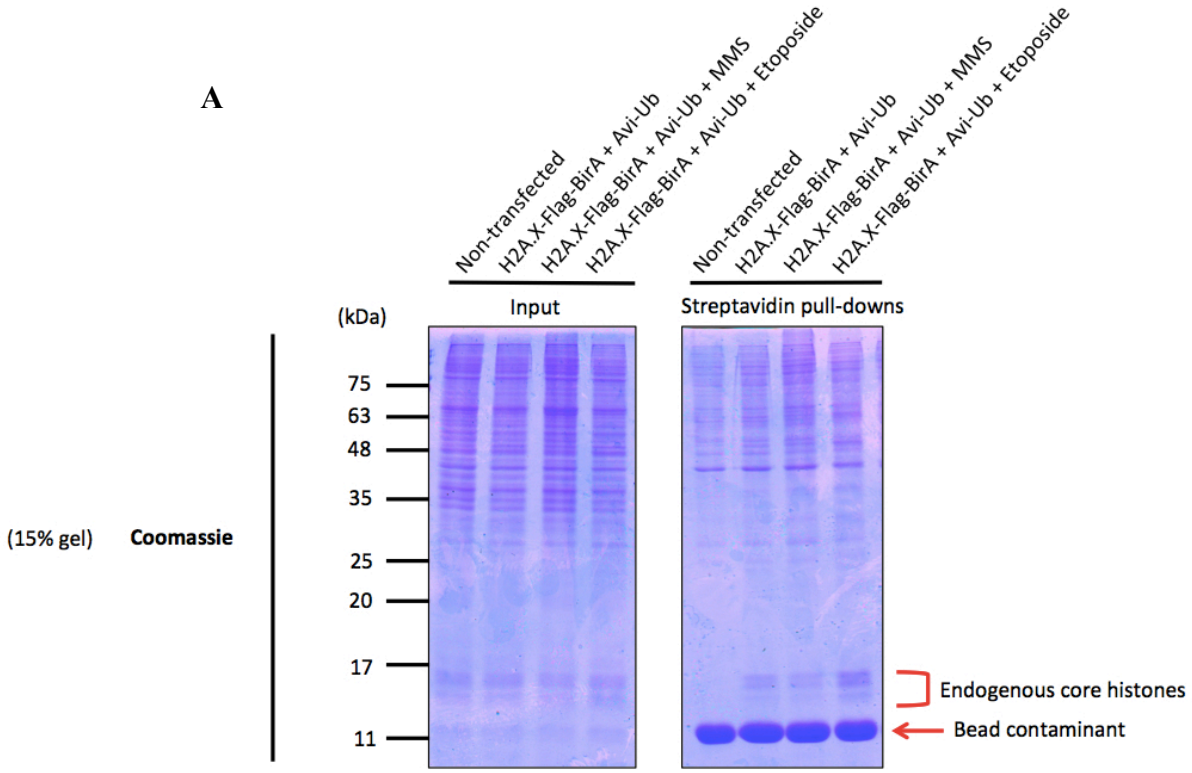
Once we verified that biotinylated substrates could effectively be pulled down with streptavidin-coupled beads, we wanted to isolate ubiquitylated H2A.X-Flag-BirA – containing nucleosomes in the presence and absence of DNA damage, as we initially sought out to do: by comparing proteins that interact with ubiquitylated H2A.X-Flag-BirA – containing nucleosomes under these two conditions, we would be able to determine which proteins interact in a DNA damage-dependent manner. We would only be able to isolate mono and di-ubiquitylated H2A.X-Flag-BirA – containing nucleosomes with streptavidin-coupled beads, as our results have suggested that polyubiquitylated forms of H2A.X-Flag-BirA are unable to become biotinylated. The streptavidin-coupled beads would also isolate the other ubiquitylated/biotinylated proteins, which may or may not be specifically associated with ubiquitylated H2A.X-Flag-BirA – containing nucleosomes. However, because these other ubiquitylated/biotinylated proteins are much less abundant than ubiquitylated/biotinylated H2A.X-Flag-BirA, the majority of proteins that are pulled down with streptavidin-coupled beads should be associated with ubiquitylated H2A.X-Flag-BirA – containing nucleosomes.

Initially, we had only planned on using one agent to induce DNA damage. However, after seeing how MMS and etoposide have different effects on the ubiquitylation of H2A.X-Flag-BirA, we thought it would be advantageous to compare proteins that interact with ubiquitylated H2A.X-Flag-BirA – containing nucleosomes in response to these two drug treatments as well. H2A.X-Flag-BirA was co-expressed with Avi-tagged ubiquitin in 293T cells, which were either left untreated, or were treated with MMS or etoposide. Nuclear lysates were prepared in a manner that would preserve protein-protein interactions. After the nuclear lysates were incubated with the streptavidin-coupled beads, bound proteins were eluted and visualized on a coomassie-stained gel (eluted proteins were loaded in equal volumes) **(Figure 21A)**. To our

surprise, many non-specific proteins bound to the beads since many proteins were eluted from non-transfected cells which should not have any biotinylated proteins. However, nucleosomes were still able to bind to the beads in a specific manner, as no endogenous core histones were eluted from the non-transfected sample. The eluted proteins were then normalized to equal levels of nucleosomes, by normalizing histone H3 levels by Western blot **(Figure 21B)**. Monoubiquitylated and di-ubiquitylated H2A.X-Flag-BirA- containing nucleosomes were pulled down, along with some of the other ubiquitylated/biotinylated proteins. This was seen when the eluted proteins were Western blotted with AVI-HRP **(Figure 21B)**. However, it was evident that monoubiquitylated H2A.X-Flag-BirA – containing nucleosomes were predominantly pulled down in each sample.

To observe differences in the eluted proteins among the different samples, the eluted proteins were resolved on an SDS-PAGE gel and stained in silver-stain for better detection as silver-stain is more sensitive than coomassie stain **(Figure 21B)**. Due to the high levels of non-specific protein binding, it is difficult to see many differences in the eluted proteins among the different samples. However, we were able to see that a protein around 20 kDa (marked by an asterisk on the gel) was found to interact with ubiquitylated H2A.X-Flag-BirA – containing nucleosomes specifically in response to both MMS and etoposide. Determining the identity of this protein would be of interest, as it may have a role in DNA damage signaling.

A


Non-transfected
H2A.X-Flag-BirA + Avi-Ub
H2A.X-Flag-BirA + Avi-Ub + MMS
H2A.X-Flag-BirA + Avi-Ub + Etoposide
Non-transfected
H2A.X-Flag-BirA + Avi-Ub
H2A.X-Flag-BirA + Avi-Ub + MMS
H2A.X-Flag-BirA + Avi-Ub + Etoposide
Input
Streptavidin pull-downs
(kDa)
75
63
48
35
25
20
17
11
(15% gel)
Coomassie
Endogenous core histones
Bead contaminant

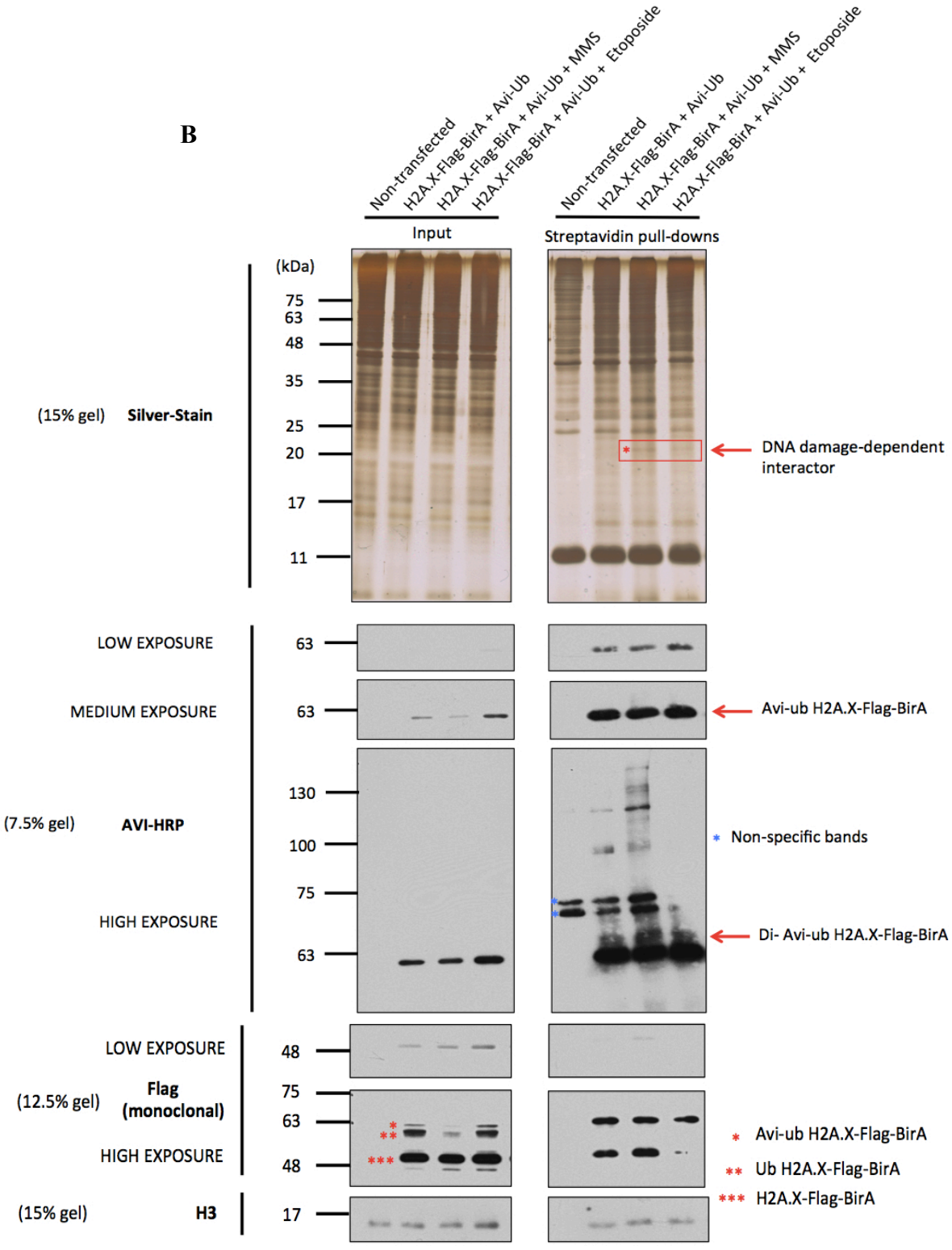

B
Non-transfected
H2A.X-Flag-BirA + Avi-Ub
H2A.X-Flag-BirA + Avi-Ub + MMS
H2A.X-Flag-BirA + Avi-Ub + Etoposide
Input
Streptavidin pull-downs
(kDa)
75
63
48
35
25
20
17
11
(15% gel) Silver-Stain
DNA damage-dependent interactor
LOW EXPOSURE
MEDIUM EXPOSURE
63
Avi-ub H2A.X-Flag-BirA
(7.5% gel) AVI-HRP
130
100
75
HIGH EXPOSURE
Non-specific bands
Di- Avi-ub H2A.X-Flag-BirA
LOW EXPOSURE
48
(12.5% gel) Flag (monoclonal)
HIGH EXPOSURE
Avi-ub H2A.X-Flag-BirA
Ub H2A.X-Flag-BirA
H2A.X-Flag-BirA
(15% gel) H3
17

FIGURE 21. Isolating ubiquitylated H2A.X-Flag-BirA – containing nucleosomes in the presence and absence of DNA damage to examine differences in protein interactors. H2A.X-Flag-BirA was co-expressed with Avi-tagged ubiquitin in 293T cells, which were either left untreated, or were treated with MMS at 300 μg/ml for 3 hours or etoposide at 60 μg/ml for 10 hours. One cell sample was left untransfected. Nuclear lysates were prepared in a manner that would preserve protein-protein interactions. Nuclear lysates were then subjected to pull-downs with streptavidin-coupled beads. **A)** Pulled-down proteins and input material (proteins that were not subjected to pull-downs) were resolved on an SDS-PAGE gel and stained in coomassie solution for visualization of all proteins (pulled-down proteins were loaded in equal volumes, and input material was loaded in equal volumes.). 0.2% of total input material and 23% of total pull-down material was loaded for each sample. **B)** Pulled-down proteins and input material were normalized to equal levels of histone H3 by western blot. After normalization, pulled-down proteins and input material were resolved on an SDS-PAGE gel and silver-stained for better visualization of proteins. Pulled-down proteins and input material were also subjected to Western blot analysis with the indicated antibodies. AVI-HRP was used to detect biotinylated substrates. The following percentages of total input material and total pull-down material were loaded for each sample: 0.08% input and 7-13% pulld-down (for silver-stained gel), 0.3% input and 8-15% pull-down (for AVI-HRP and Flag blots) and 0.04% input and 3-7% pull-down (for H3 blot). Ub = ubiquitin/ubiquitylated. n =1.

3.2 Elucidating the Relationship Between H2A.X Monoubiquitylation at K118/119, H2A.X Monoubiquitylation at K13/15, and H2A.X Phosphorylation at S139 During DNA Damage

3.2.1 Examining the Ubiquitylation and Phosphorylation of Various H2A.X-Flag-BirA Mutants in the Presence and Absence of DNA Damage

To gain a better understanding of the relationship between H2A.X monoubiquitylation at K118/119, H2A.X monoubiquitylation at K13/15, and H2A.X phosphorylation at S139, we constructed plasmids to express the following point mutants: H2A.X-K118/119R-Flag-BirA, H2A.X-K13/15R-Flag-BirA, H2A.X-K118/119R, K13/15R-Flag-BirA and H2A.X-S139A-Flag-

BirA. A lysine to arginine mutation ablates ubiquitylation, and a serine to alanine mutation ablates phosphorylation. To examine the effects each mutation has on the ubiquitylation and phosphorylation of H2A.X, WT H2A.X-Flag-BirA and each of the mutant forms were co-expressed with Avi-tagged ubiquitin in 293T cells in both the presence and absence of DNA damage. MMS was used to induce DNA damage in one set **(Figure 22)**, and etoposide was used to induce DNA damage in another set **(Figure 23)**. Nuclear lysates were prepared and subjected to Western blot analysis.

The data suggest that monoubiquitylation at K118/119 is not required for phosphorylation at S139, as levels of phosphorylation on the K118/119R mutant appeared equal to phosphorylation levels on the WT form, in response to both MMS and etoposide. It is difficult to determine if phosphorylation at S139 is required for monoubiquitylation at K13/15, since it was difficult to detect monoubiquitylation at K13/15 on the WT form for comparison, even in the presence of MMS or etoposide. Monoubiquitylation at K118/119 doesn't appear to be a prerequisite for monoubiquitylation at K13/15, as monoubiquitylation at K13/15 was seen on the K118/119R mutant in the absence of DNA damage (seen on the Flag and AVI-HRP blots from both sets at high exposures). Monoubiquitylation at K13/15 on the K118/119R mutant decreased in response to MMS and increased in response to etoposide (seen on the Flag and AVI-HRP blots at high exposures). Apart from K118/119 and K13/15, it appears H2A.X can be ubiquitylated at other sites since higher bands were seen above the unmodified form of the K118/119R, K13/15R mutant; however, they were present only at low levels (seen on the Flag and AVI-HRP blots from both sets at high exposures). Additionally, it appears that neither monoubiquitylation at K118/119, monoubiquitylation at K13/15, or phosphorylation at S139 is required for ubiquitylation of the other biotinylated proteins: for each mutant form of H2A.X-

Flag-BirA, ubiquitylation of the other proteins was still seen to increase in response to MMS and etoposide, seen as smears of bands above 63 kDa (seen on the AVI-HRP blots from both sets at high exposures).

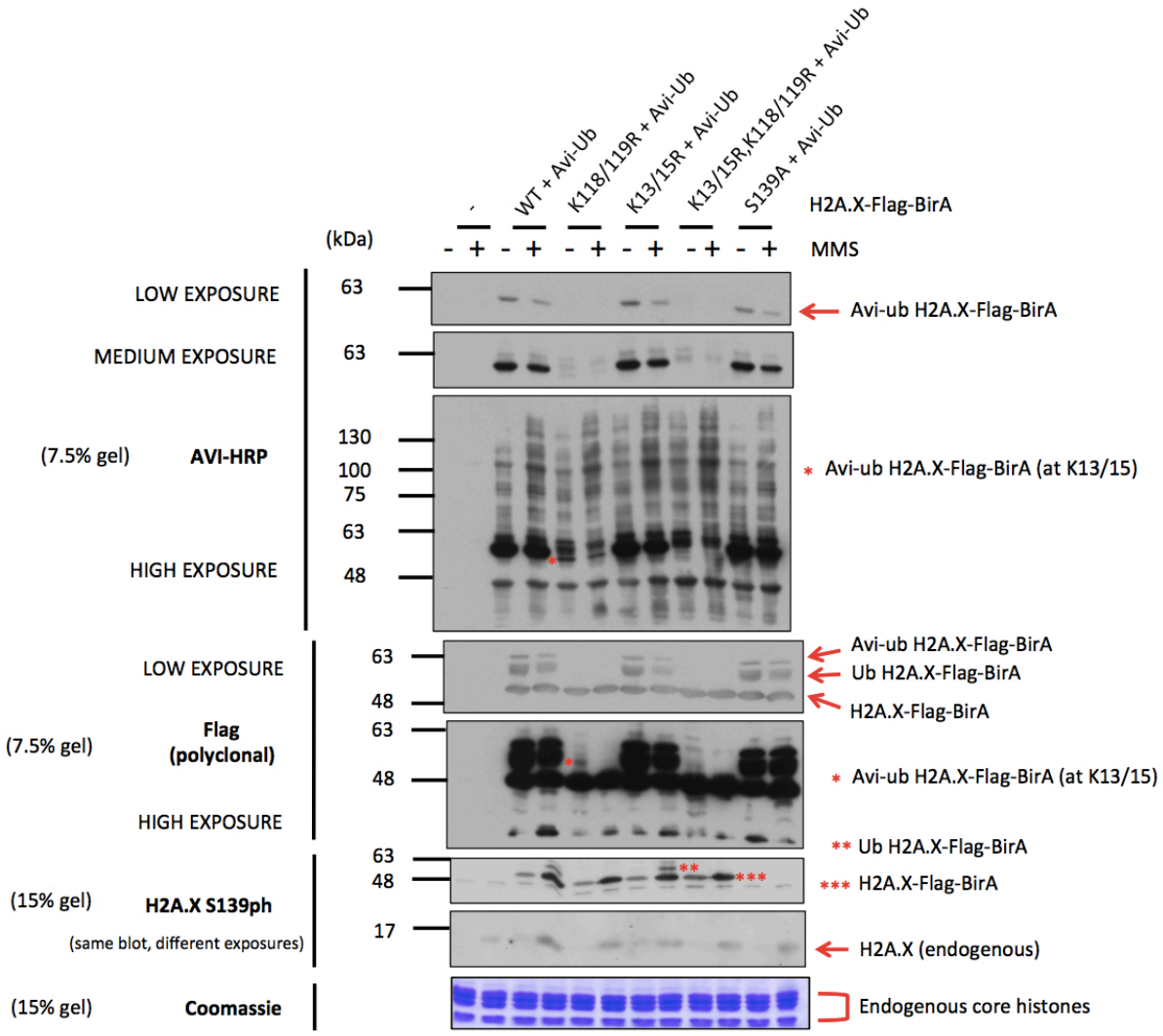


FIGURE 22. Examining the ubiquitylation and phosphorylation of various H2A.X-Flag-BirA mutants in the presence and absence of MMS. Avi-tagged ubiquitin was co-expressed with H2A.X-Flag-BirA, H2A.X-K118/119R-Flag-BirA, H2A.X-K13/15R-Flag-BirA, H2A.X-K118/119R, K13/15R-Flag-BirA or H2A.X-S139A-Flag-BirA in 293T cells, and treated with MMS at 300 µg/ml for 3 hours, or sterile water for 3 hours (the solvent MMS was dissolved in, used as a control). Additionally, one untransfected cell sample was treated with MMS, and another untransfected cell sample was treated with sterile water. Nuclear lysates were prepared, and proteins were resolved on SDS-PAGE gels and subjected to Western blot analysis with the indicated antibodies. AVI-HRP was used to detect biotinylated substrates, and H2A.X S139 phosphorylation was used to assess levels of DNA DSBs. Below the Western blots is a coomassie-stained gel showing the relative levels of the endogenous core histones present in the nuclear lysates, which acted as loading controls. Ub = ubiquitin/ubiquitylated. n = 1.

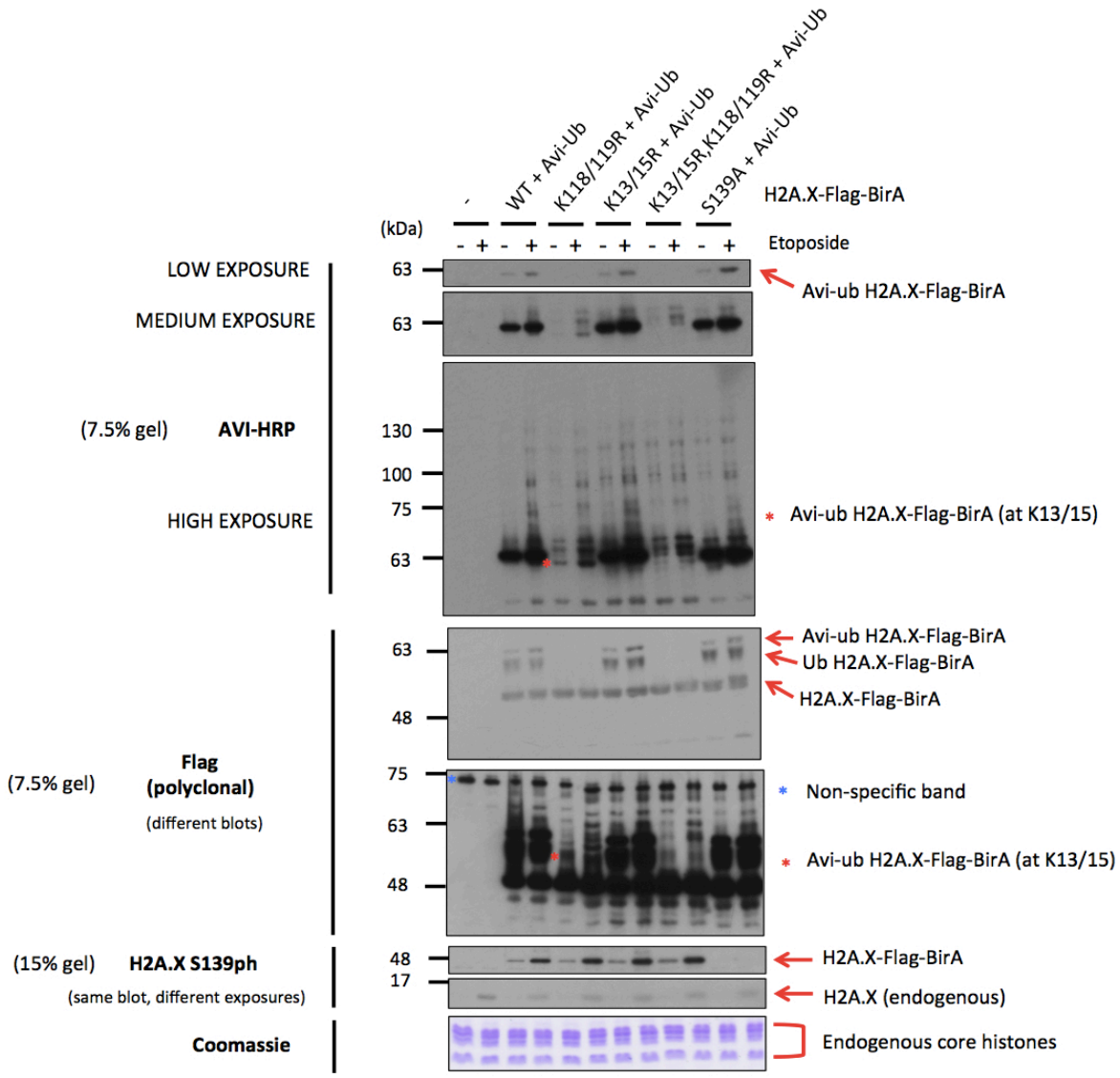


FIGURE 23. Examining the ubiquitylation and phosphorylation of various H2A.X-Flag-BirA mutants in the presence and absence of etoposide. Avi-tagged ubiquitin was co-expressed with H2A.X-Flag-BirA, H2A.X-K118/119R-Flag-BirA, H2A.X-K13/15R-Flag-BirA, H2A.X-K118/119R, K13/15R-Flag-BirA or H2A.X-S139A-Flag-BirA in 293T cells, and treated with etoposide at 60 µg/ml for 10 hours, or DMSO for 10 hours (the solvent etoposide was dissolved in, used as a control). Additionally, one untransfected cell sample was treated with etoposide, and another untransfected cell sample was treated with DMSO. Nuclear lysates were prepared, and proteins were resolved on SDS-PAGE gels and subjected to Western blot analysis with the indicated antibodies. AVI-HRP was used to detect biotinylated substrates, and H2A.X S139 phosphorylation was used to assess levels of DNA DSBs. Below the Western blots is a coomassie-stained gel showing the relative levels of the endogenous core histones present in the nuclear lysates, which acted as loading controls. Ub = ubiquitin/ubiquitylated. n =1.

3.2.2 Analyzing the Effects of RNF168 Overexpression on the Ubiquitylation of H2A.X-Flag-BirA

We have seen that, unlike monoubiquitylation of H2A.X-Flag-BirA at K118/119, monoubiquitylation of H2A.X-Flag-BirA at K13/15 is not easily detectable, even in the presence of DNA damage. Therefore, we wanted to see if we could increase levels of monoubiquitylation at this site by overexpressing the E3 ubiquitin ligase RNF168, which is reported to be responsible for monoubiquitylation on H2A.X/H2A at K13/15 (Mattiroli et al., 2012). To test this, H2A.X-Flag-BirA was co-expressed with Avi-tagged ubiquitin in 293T cells, with or without an HA-tagged RNF168 construct. Nuclear lysates were prepared and subjected to Western blot analysis **(Figure 24)**. We saw that overexpressing HA-RNF168 did increase monoubiquitylation at K13/15, as there was an increase in di-ubiquitylated H2A.X-Flag-BirA and to a lesser extent, tri-ubiquitylated H2A.X-Flag-BirA (seen on the Flag and AVI-HRP blots at high exposure). Presumably, the di-ubiquitylated form is monoubiquitylated at either K13 or K15, and the tri-ubiquitylated form is either simultaneously monoubiquitylated at both K13 and K15, or is di-ubiquitylated at either K13 or K15.

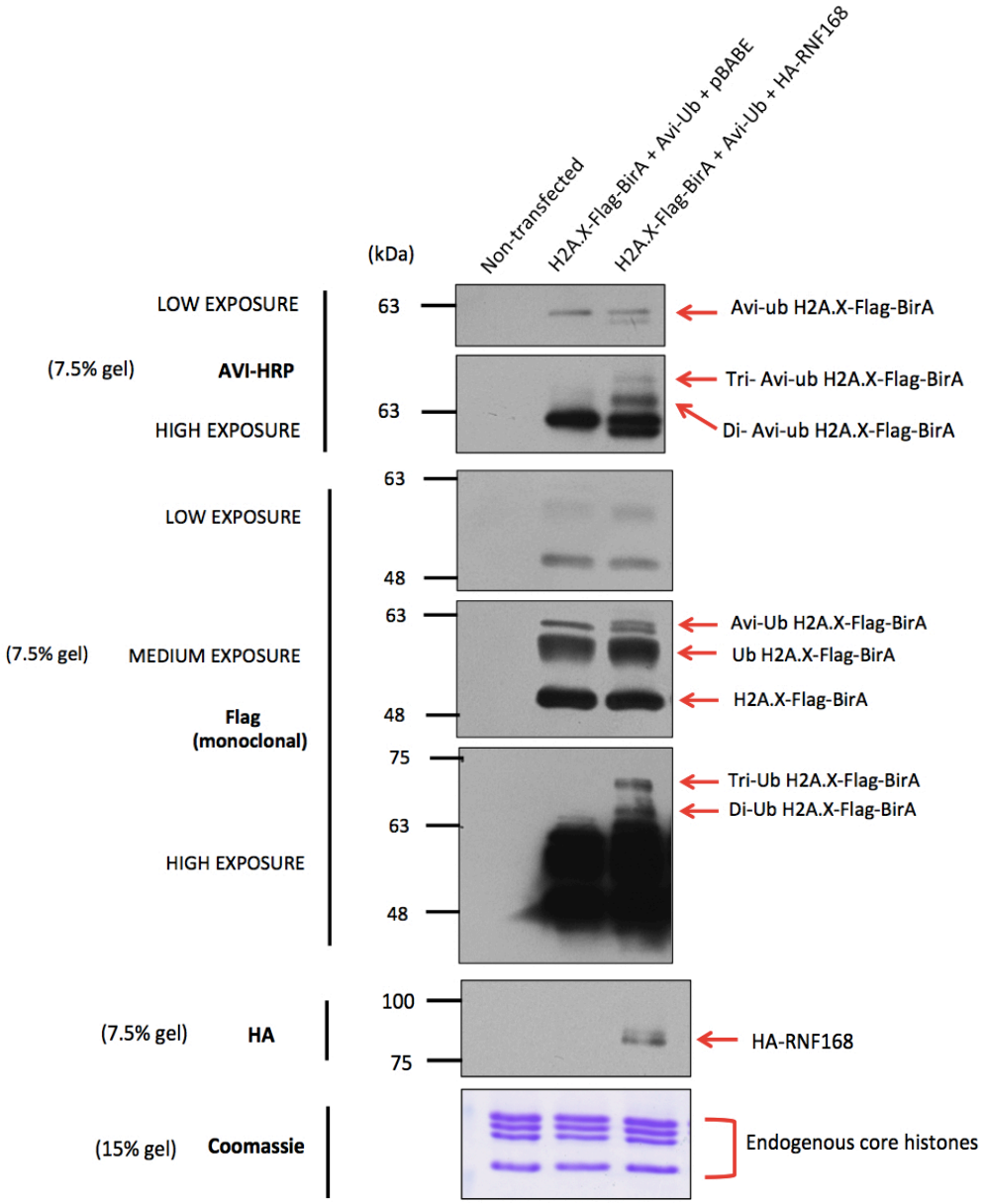


FIGURE 24. Examining the effects of RNF168 overexpression on the ubiquitylation of H2A.X-Flag-BirA. H2A.X-Flag-BirA was co-expressed with Avi-tagged ubiquitin in 293T cells with either HA-RNF168 or an empty pBABE vector. The pBABE vector was expressed in the absence of HA-RNF168 to normalize the total amount of transfected plasmids between the two samples. One cell sample was left untransfected. Nuclear lysates were prepared, and proteins were resolved on SDS-PAGE gels and subjected to Western blot analysis with the indicated antibodies. AVI-HRP was used to detect biotinylated substrates. Below the Western blots is a coomassie-stained gel showing the relative levels of the endogenous core histones present in the nuclear lysates, which acted as loading controls. Transfections, cell harvesting and preparation of nuclear lysates were done by Alegria Indio. Ub = ubiquitin/ubiquitylated. n =1.

Although K13/15 has been reported to be the site of RNF168 ubiquitylation on H2A.X/H2A, we wanted to verify that the ubiquitylation we saw on H2A.X-Flag-BirA with RNF168 overexpression was indeed at this site. We also wanted to examine the effects of RNF168 overexpression on the various H2A.X-Flag-BirA mutants. H2A.X-Flag-BirA, H2A.X-K118/119R-Flag-BirA, H2A.X-K13/15R-Flag-BirA, H2A.X-K118/119R, K13/15R-Flag-BirA and H2A.X-S139A-Flag-BirA were either singly expressed or co-expressed with HA-RNF168 in 293T cells. Nuclear lysates were prepared and subjected to Western blot analysis **(Figure 25)**. We were able to verify that K13/15 was the site of RNF168 ubiquitylation, as HA-RNF168 was seen to increase di-ubiquitylation on H2A.X-Flag-BirA, but not on H2A.X-K13/15R-Flag-BirA. Additionally, we saw that H2A.X doesn't have to be phosphorylated at S139 in order for RNF168 to ubiquitylate it at K13/15: HA-RNF168 was still able to increase di-ubiquitylation on H2A.X-S139A-BirA. Besides K13/15, RNF168 does not appear to ubiquitylate H2A.X on any other sites: monoubiquitylation at K118/119 on H2A.X-K13/15R-Flag-BirA was not increased with HA-RNF168 overexpression, and no ubiquitylation was visible on H2A.X-K118/119R, K13/15R-Flag-BirA, even with HA-RNF168 overexpression. Interestingly, monoubiquitylation levels at K13/15 were higher on H2A.X-K118/119R-Flag-BirA compared to H2A.X-Flag-BirA in response to HA-RNF168 overexpression. This observation is seen on the Flag blot at high exposure, where ubiquitylated H2A.X-K118/119R-Flag-BirA (lane 5) is stronger than di-ubiquitylated H2A.X-Flag-BirA (lane 3). This finding indicates that the presence of ubiquitin at K118/119 on H2A.X somehow decreases the ability for H2A.X to be ubiquitylated at K13/15. To our knowledge, this has not been reported, and it could be interesting since it suggests a potential antagonism between K118/119 ubiquitylation and K13/15 ubiquitylation.

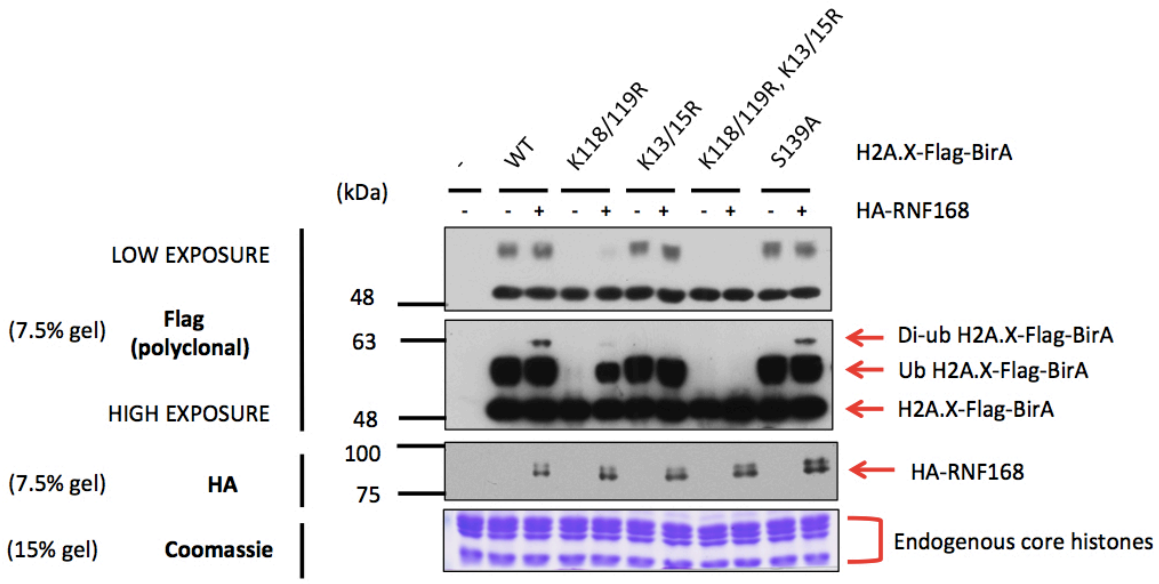


FIGURE 25. Examining the effects of RNF168 overexpression on the ubiquitylation of various H2A.X-Flag-BirA mutants. H2A.X-Flag-BirA, H2A.X-K118/119R-Flag-BirA, H2A.X-K13/15R-Flag-BirA, H2A.X-K118/119R, K13/15R-Flag-BirA and H2A.X-S139A-Flag-BirA were individually co-expressed with either HA-RNF168 or an empty pBABE vector in 293T cells. The pBABE vector was expressed in the absence of HA-RNF168 to normalize the total amount of transfected plasmids between the samples. One cell sample was left untransfected. Nuclear lysates were prepared, and proteins were resolved on SDS-PAGE gels and subjected to Western blot analysis with the indicated antibodies. Below the Western blots is a coomassie-stained gel showing the relative levels of the endogenous core histones present in the nuclear lysates, which acted as loading controls. Ub = ubiquitin/ubiquitylated. n = 1.

One reason why ubiquitylation at K118/119 might decrease the RNF168-induced K13/15 ubiquitylation of H2A.X could be due to physical hindrance of the binding of RNF168 to H2A.X. To test this, we examined the amount of RNF168 that co-IPs with WT vs. K118/119R H2A.X-Flag-BirA – containing nucleosomes. H2A.X-Flag-BirA and H2A.X-K118/119R-Flag-BirA were individually expressed in 293T cells with HA-RNF168. Nuclear lysates were prepared in a manner that would preserve protein-protein interactions. Nuclear lysates were

subjected to immunoprecipitation with anti-Flag antibodies, and the immunoprecipitated proteins were subjected to western blot analysis **(Figure 26)**. The results showed that equal amounts of RNF168 were co-purified with WT or K118/119R H2A.X-Flag-BirA- containing nucleosomes. Therefore, it is unlikely that ubiquitylation at K118/119 hinders the ability of RNF168 to bind to H2A.X, as we had hypothesized.

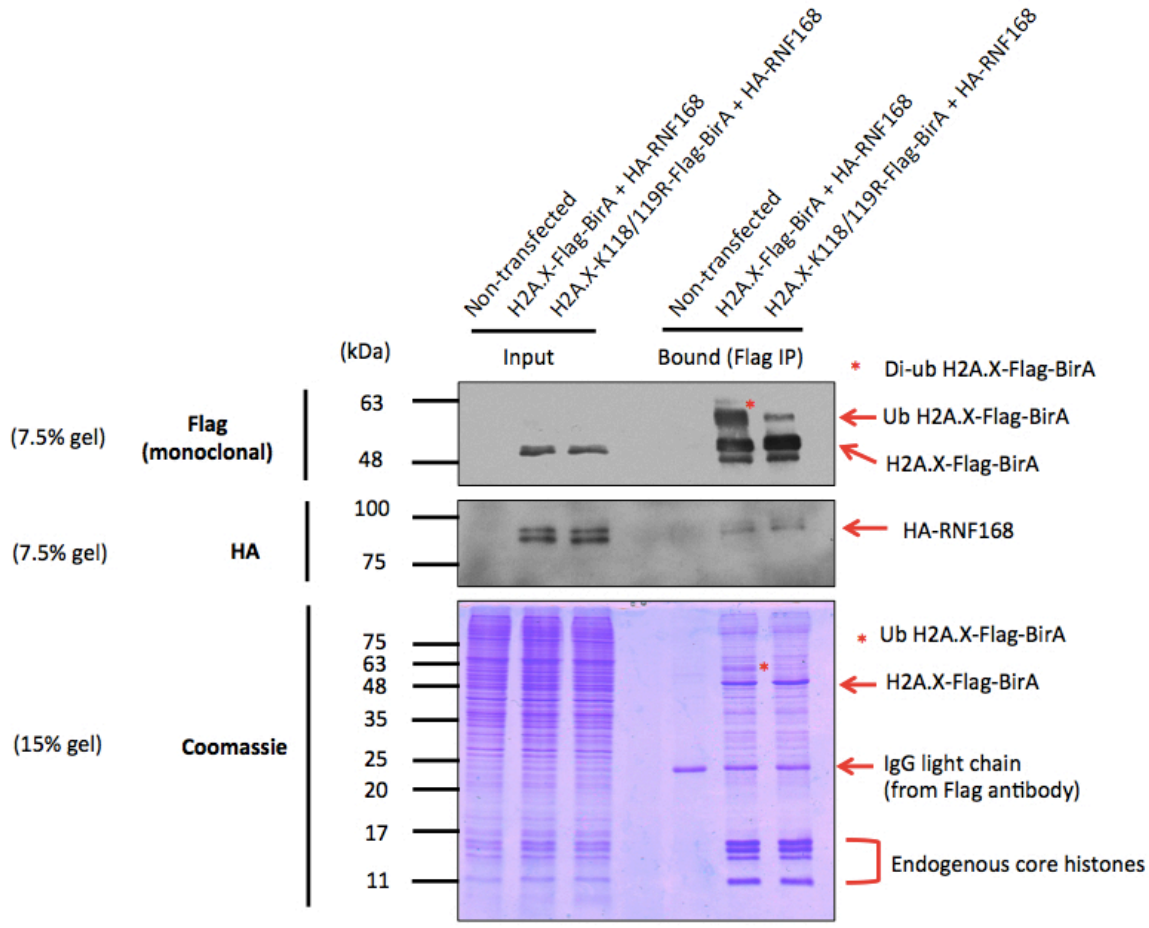


FIGURE 26. Comparing the interaction of RNF168 with H2A.X-Flag-BirA versus H2A.X-K118/119R-Flag-BirA. HA-RNF168 was either co-expressed with H2A.X-Flag-BirA or H2A.X-K118/119R-Flag-BirA in 293T cells. One cell sample was left untransfected. Nuclear lysates were prepared in a manner that would preserve protein-protein interactions. Nuclear lysates were then subjected to immunoprecipitation with anti-Flag antibodies. Immunoprecipitated proteins (bound fraction) and input material (proteins that were not subjected to immunoprecipitation) were subjected to Western blot analysis with the indicated antibodies. Immunoprecipiated proteins and input material were also resolved on an SDS-PAGE gel and stained in coomassie solution for visualization of all proteins. 0.2% of total input material and 13% of total immunoprecipitated material was loaded for each sample for western blots, and 0.4% of total input material and 25% of total immunoprecipitated material was loaded for the coomassie-stained gel. Ub = ubiquitin/ubiquitylated. n =1.

CHAPTER 4: DISCUSSION

The main objective of this project was to gain a better understanding of the mechanisms associated with H2A.X monoubiquitylation marks during DNA DSB signaling. In an effort to do so, we wanted to do two things: 1) Identify proteins that interact with ubiquitylated H2A.X – containing nucleosomes in a DNA damage – dependent manner, and 2) Elucidate the relationship between H2A.X monoubiquitylation at K118/119, H2A.X monoubiquitylation at K13/15, and H2A.X phosphorylation at S139 during DNA damage. Here we discuss our experimental findings.

4.1 Identifying Proteins that Interact with Ubiquitylated H2A.X – Containing Nucleosomes in a DNA Damage - Dependent Manner

In order to identify proteins that interact with ubiquitylated H2A.X- containing nucleosomes, we sought to develop a system that would allow us to specifically tag ubiquitylated H2A.X histones with biotin *in vivo*. This system involves the co-expression of an H2A.X-Flag-BirA fusion protein with Avi-tagged ubiquitin in mammalian cells. The assumption is, if H2A.X-Flag-BirA becomes ubiquitylated with Avi-tagged ubiquitin, the BirA would preferentially catalyze the addition of a biotin molecule to the Avi-tagged ubiquitin due to the physical proximity of Avi-tagged ubiquitin and the BirA portion of H2A.X-Flag-BirA, and also because both the enzyme and substrate are now on the same histone molecule. Once the ubiquitylated H2A.X-Flag-BirA proteins are tagged with biotin, ubiquitylated H2A.X-Flag-BirA – containing nucleosomes could then be purified using streptavidin-coupled beads. Proteins associated with the ubiquitylated H2A.X-Flag-BirA – containing nucleosomes could also be co-purified and identified using mass spectrometry. Furthermore, we aimed to perform the co-

purification from cells exposed or not exposed to DNA-damaging agents to further distinguish and identify DNA-damage-dependent interactions.

As proof of principle, we first co-expressed H2A.X-Flag-BirA and Avi-tagged ubiquitin in 293T cells in the absence of DNA damage and confirmed that the H2A.X-Flag-BirA was able to become biotinylated when monoubiquitylated with Avi-tagged ubiquitin at the K118/119 site. We expected to see monoubiquitylation of H2A.X in the absence of induced DNA damage, since a fraction of H2A and H2A.X histones are known to be monoubiquitylated on K118/119 at sites of transcriptional repression (Mattiroli et al., 2012; Wu et al., 2011). Once we confirmed that monoubiquitylated H2A.X-Flag-BirA at K118/119 could become biotinylated, we next sought to test the ubiquitylation and biotinylation of H2A.X-Flag-BirA in response to DNA damage. We first examined the efficiencies of different DNA damage – inducing agents and determined that MMS, etoposide, doxorubicin, and the Cas9-Alu plasmid were able to effectively induce DSBs in cells. After optimizing each of these agents and collectively comparing their abilities to induce DSBs, we determined that MMS was the most efficient DNA damaging agent since it induced the highest levels of H2A.X S139 phosphorylation. The other three agents appeared to be less effective to induce H2A.X S139 phosphorylation.

Despite any differences in the abilities of the agents to induce DSBs, we anticipated that each of them would have similar effects on the ubiquitylation of H2A.X-Flag-BirA, although perhaps to different extents. We expected there to be an increase in monoubiquitylation at the K118/119 site (Pan et al., 2011; Wu et al., 2011) and an increase in monoubiquitylation and polyubiquitylation at the K13/15 site (Mattiroli et al., 2012), as these ubiquitylation marks have been reported to occur on H2A.X in response to DSBs. To our surprise, we observed differences in the ubiquitylation of H2A.X-Flag-BirA in response to the different DSB – inducing agents

(Table 1). An increase in monoubiquitylation at the K118/119 site was seen in response to etoposide and doxorubicin, whereas a decrease in monoubiquitylation at the K118/119 site was seen in response to MMS and expression of the Cas9-Alu plasmid. An increase in polyubiquitylation of H2A.X-Flag-BirA was seen in response to MMS, etoposide and doxorubicin, but not in response to the expression of the Cas9-Alu plasmid. Finally, we saw that monoubiquitylation at the K13/15 site increased in response to etoposide, but decreased in response to MMS. Regardless of the different effects each agent had on the ubiquitylation of H2A.X-Flag-BirA, monoubiquitylation levels on K118/119 were always much higher than monoubiquitylation and/or polyubiquitylation levels on K13/15.

TABLE 1. A summary of the effects different DNA damage-inducing agents had on the ubiquitylation of H2A.X-Flag-BirA.

Agent	Effect on Monoubiquitylation at K118/119	Effect on Monoubiquitylation at K13/15	Effect on Polyubiquitylation (Presumed to be at K13/15)
MMS	Decrease	Decrease	Increase
Etoposide	Increase	Increase	Increase
Doxorubicin	Increase	N/A	Increase
Cas9-Alu Plasmid	Decrease	N/A	No Effect

Some of the effects we have seen with these DNA damage – inducing agents have also been seen by other researchers. For example, monoubiquitylation of H2A at the K13/15 site was seen to increase in response to etoposide in a study conducted by Penengo and colleagues (Gatti et al., 2012). Another study showed that monoubiquitylation of H2A at the K118/119 site

decreased in response to MMS (Nakata et al., 2016). The majority of studies that have analyzed H2A/H2A.X ubiquitylation during DNA damage have done so in response to IR, which increases its monoubiquitylation at the K118/119 site (Pan et al., 2011; Wu et al., 2011) and at the K13/15 site (Leung et al., 2014; Mattiroli et al., 2012; Wang et al., 2016). IR directly induces DSBs (Borrego-Soto et al., 2015). Based on our's and other's findings, we can group the different DNA damage – inducing agents into two categories: those that induce monoubiquitylation at the K118/119 and/or K13/15 site (IR, etoposide, doxorubicin) and those that decrease monoubiquitylation at the K118/119 and/or K13/15 site (MMS and the Cas9-Alu plasmid). Although each of these agents ultimately induces the formation of DSBs, they evidently affect ubiquitylation of H2A and H2A.X in different ways. It is unclear why these differences in ubiquitylation are seen, as there doesn't seem to be a common mechanism associated with the agents within each category. In fact, agents in different categories share similarities: IR (Borrego-Soto et al., 2015) and Cas9 (Ran et al., 2013) directly induce DSBs, whereas MMS (Staszewski et al., 2008) and etoposide (Muslimović et al., 2008) indirectly induce DSBs during DNA replication (MMS adds alkyl groups to bases and etoposide generates transient SSBs and transient DSBs, all of which can result in the formation of stable DSBs during replication). Nevertheless, the fact that these agents have different effects on the ubiquitylation of H2A/H2A.X is of interest because it suggests that the signaling mechanisms associated with DSB repair may vary depending on the DSB-inducing agent. In other words, there may not be just one signaling pathway that is uniform in response to the induction of all DSBs. This is important to consider, since most studies that examine H2A/H2A.X ubiquitylation or other elements of DSB repair do so in response to IR. Therefore, not all of the proteins and PTMs reported to be involved in DSB signaling may pertain to all types of DSBs. It is also

possible that the differences in H2A.X ubiquitylation seen in response to the different DNA damage-inducing agents are a result of potential differences in cell cycle regulation. If the different agents cause cells to be in different cell cycle phases, ubiquitylation/E3 ligase activity may consequently be different as well. None of the studies described above showed increases in polyubiquitylated forms of H2A/H2A.X in response to DNA damage (by IR, etoposide or MMS). However, one of the studies showed that levels of polyubiquitylated H2A were increased in response to RNF168 overexpression (Mattiroli et al., 2012). They were able to show that polyubiquitylation on H2A occurred at the K13/15 site, the polyubiquitin chains were K63- linked, and formation of the polyubiquitin chains required the presence of endogenous RNF8. Therefore, it is likely that the induction of polyubiquitylation we saw on H2A.X-Flag-BirA in response to MMS, etoposide and doxorubicin required the activities of RNF168 and RNF8.

From our experiments, we were able to see that H2A.X-Flag-BirA was able to become biotinylated when solely monoubiquitylated with Avi-tagged ubiquitin at the K13/15 site. We also saw that di-ubiquitylated and tri-ubiquitylated forms of H2A.X-Flag-BirA could also become biotinylated. Therefore, in addition to monoubiquitylated H2A.X-Flag-BirA at the K118/119 site, we have the potential of isolating these other ubiquitylated forms of H2A.X-Flag-BirA with streptavidin-coupled beads. We were not able to detect any polyubiquitylated forms of H2A.X-Flag-BirA that were biotinylated. It is possible that ubiquitin on polyubiquitin chains are sterically unfavourable to be biotinylated by BirA on the same molecule. Alternatively, it is possible that polyubiquitylated forms of H2A.X-Flag-BirA are biotinylated, but difficult to detect. We saw that levels of polyubiquitylation on H2A.X-Flag-BirA in response to MMS, etoposide and doxorubicin were very low, and since fewer substrates are ubiquitylated with Avi-

tagged ubiquitin opposed to endogenous ubiquitin, the amount of polyubiquitylated H2A.X-Flag-BirA with Avi-tagged ubiquitin that is biotinylated may be too low to detect.

Apart from ubiquitylated forms of H2A.X-Flag-BirA itself, we found that H2A.X-Flag-BirA was also able to biotinylate other proteins that were ubiquitylated with Avi-tagged ubiquitin. However, levels of these other biotinylated proteins were very low relative to levels of biotinylated/monoubiquitylated H2A.X-Flag-BirA at K118/119. The fact that these other biotinylated proteins are much lower in abundance compared to biotinylated H2A.X-Flag-BirA suggests that the system is working – that autobiotinylation of ubiquitylated H2A.X-Flag-BirA is much higher than biotinylation of other ubiquitylated proteins. Since BirA biotinylation activity is supposed to be proximity-dependent, these proteins are likely to be associated with H2A.X in some way, either by direct or indirect interaction. Although we weren't able to determine if these proteins are specifically associated with H2A.X, we were able to verify that these proteins are associated with nucleosomes. Ubiquitylation of these other proteins was seen to increase in response to MMS, etoposide and doxorubicin, although the highest increases were seen with MMS, possibly because MMS induced the formation of more DSBs. This finding was not surprising, since many ubiquitylation targets have been linked to DSB signaling and repair.

We saw that the MMS/etoposide – induced increase in ubiquitylation of the other biotinylated proteins was similar among cells that expressed WT H2A.X-Flag-BirA, H2A.X-K118/119R, K13/15R-Flag-BirA and H2A.X-S139A-Flag-BirA. Therefore, at first glance, these results suggest that ubiquitylation of these proteins is not dependent on H2A.X ubiquitylation at K118/119 or K13/15, or H2A.X phosphorylation at S139. However, because every nucleosome has two copies of an H2A-type histone, nucleosomes containing a mutant copy of H2A.X-Flag-BirA may also contain an endogenous copy of WT H2A.X or H2A. Because of this caveat, we

cannot conclude with certainty that ubiquitylation of these proteins is not dependent on H2A.X ubiquitylation and/or phosphorylation. It is unlikely that H2A.X monoubiquitylation at K118/119 and K13/15 are important for the ubiquitylation of these proteins, since monoubiquitylation at these two sites decreases in response to MMS. However, since H2A.X phosphorylation at S139 is seen to increase alongside the increase in ubiquitylation of these proteins, it is possible that H2A.X phosphorylation at S139 is required for the ubiquitylation of these proteins. Regardless, the fact that we see increases in the ubiquitylation of these proteins in response to MMS, etoposide and doxorubicin suggests that these ubiquitylated proteins may play important roles during DSB signaling and repair, at least for these particular DSB – inducing agents. Therefore, the fact that these ubiquitylated proteins are also able to become biotinylated is advantageous, as we also have the potential of isolating these proteins with streptavidin-coupled beads and determining their identities using mass spectrometry. We have already seen that these biotinylated/ubiquitylated proteins can be pulled down using streptavidin-coupled beads.

As previously mentioned, we initially thought that in response to any DNA DSB – inducing agent, we would see increases in monoubiquitylation at K118/119, monoubiquitylation at K13/15, and polyubiquitylation at K13/15 on H2A.X-Flag-BirA. Therefore, we expected to isolate more monoubiquitylated (at K118/119), di-ubiquitylated (monoubiquitylated at K118/119 and K13/15), and polyubiquitylated H2A.X-Flag-BirA – containing nucleosomes with streptavidin-coupled beads from a DNA damage – induced sample compared to a non DNA-damage – induced sample. In this manner, if mass spectrometry were used to identify co-purified proteins from the two conditions, we would be able to identify proteins that are associated with these ubiquitylation marks on H2A.X in response to DSBs. However, we have

seen that different DSB – inducing agents have different effects on H2A.X ubiquitylation. For example, although etoposide and MMS both increase levels of polyubiquitylated H2A.X, etoposide increases levels of monoubiquitylation at the K118/119 and K13/15 sites, while MMS decreases levels of monoubiquitylation at these sites. After observing these differences, we wanted to isolate ubiquitylated H2A.X-Flag-BirA – containing nucleosomes under both MMS and etoposide – treated conditions. By doing this, we could compare proteins that interact with ubiquitylated H2A.X-Flag-BirA – containing nucleosomes in response to these two different DNA – damage inducers.

Despite the differences in H2A.X ubiquitylation between MMS and etoposide, under both treatments, levels of monoubiquitylated H2A.X-Flag-BirA at K118/119 are much higher than levels of di-ubiquitylated and polyubiquitylated H2A.X-Flag-BirA. The relative difference in abundance between monoubiquitylated H2A.X-Flag-BirA at K118/119 and the other ubiquitylated forms is so great that, if all ubiquitylated H2A.X-Flag-BirA – containing nucleosomes were purified, the amounts of di-ubiquitylated and polyubiquitylated H2A.X-Flag-BirA – containing nucleosomes would be negligible compared to the amount of monoubiquitylated H2A.X-Flag-BirA-containing nucleosomes. Therefore, we are limited to isolating and examining only monoubiquitylated H2A.X-Flag-BirA – containing nucleosomes at the K118/119 site under both MMS and etoposide-treated conditions. As a result, we have the potential of identifying proteins that interact with monoubiquitylated H2A.X-Flag-BirA-containing nucleosomes in response to MMS and etoposide. However, the recruitment of these potential proteins to nucleosomes may or may not be dependent on the monoubiquitylation mark at K118/119 (i.e some proteins may bind to nucleosomes in response to MMS and etoposide independent of monoubiquitylation on H2A.X at K118/119).

When we isolated monoubiquitylated H2A.X-Flag-BirA – containing nucleosomes with streptavidin-coupled beads under normal, MMS-treated and etoposide-treated conditions, we found that a protein around 20 kDa was co-purified under MMS-treated and etoposide-treated conditions, but not under normal conditions. These findings suggest that this particular protein interacts with nucleosomes in response to the induction of DSBs caused by MMS and etoposide. It would be advantageous to determine the identity of this protein, since it may be a protein that hasn't yet been discovered to play a role in DSB signaling and repair. Unfortunately, we weren't able to detect any other differences in co-purified proteins among the three different conditions, due to high levels of non-specific protein binding to the streptavidin-coupled beads. Once we determine a way of removing this non-specific protein binding, we will hopefully be able to see more differences in co-purified proteins among the different conditions.

4.2 Elucidating the Relationship Between H2A.X Monoubiquitylation at K118/119, H2A.X Monoubiquitylation at K13/15, and H2A.X Phosphorylation at S139 During DNA Damage

To gain a better understanding of the relationship between H2A.X monoubiquitylation at K118/119, H2A.X monoubiquitylation at K13/15, and H2A.X phosphorylation at S139 during DNA damage, we made the following mutant H2A.X-Flag-BirA constructs: H2A.X-K118/119R-Flag-BirA, H2A.X-K13/15R-Flag-BirA, H2A.X-K118/119R, K13/15R-Flag-BirA and H2A.X-S139A-Flag-BirA. WT and mutant H2A.X-Flag-BirA constructs were individually expressed in 293T cells, which were either subjected to DNA damage (by MMS or etoposide) or left untreated. Potential changes in ubiquitylation and phosphorylation seen with each mutant H2A.X-Flag-BirA protein were examined.

Our results showed that H2A.X monoubiquitylation at K118/119 was not required for H2A.X phosphorylation at S139, since in response to both MMS and etoposide, phosphorylation levels on H2A.X-K118/119R-Flag-BirA were similar to levels of phosphorylation on WT H2A.X-Flag-BirA. This finding was unexpected, since two studies have reported H2A.X monoubiquitylation at K118/119 to be a prerequisite for H2A.X phosphorylation at S139 (Pan et al., 2011; Wu et al., 2011). The authors of those studies proposed that monoubiquitylaion at K118/119 helps recruit the ATM kinase for phosphorylation. Experiments from both studies showed that H2A.X phosphorylation was decreased (but still present) on an H2A.X K118/119R mutant compared to WT H2A.X in response to IR. However, it is important to note that these experiments were done using H2A.X null mouse embryonic fibroblasts ($H2A.X^{-/-}$ MEFs) that were stably re-constituted with either H2A.X K118/119R or WT H2A.X. In our experiments, we expressed H2A.X-K118/119R-Flag-BirA in 293T cells that still contained endogenous H2A.X, whereas in their experiments, they were able to stably express H2A.X K118/119R in cells without endogenous H2A.X. Therefore, it is possible that the reason we didn't see a decrease in phosphorylation on H2A.X-K118/119R-Flag-BirA is because monoubiquitylation of endogenous H2A.X at K118/119 was able to compensate *in trans* for lost monoubiquitylation on H2A.X-K118/119R-Flag-BirA. The fact that some H2A.X K118/119R histones could still become phosphorylated in $H2A.X^{-/-}$ MEFs also suggests that monoubiquitylation of endogenous H2A at K118/119 might compensate for lost monoubiquitylation on H2A.X. The notion that H2A ubiquitylation may be able to compensate for the lack of H2A.X ubiquitylation was also proposed by Leung et al (Leung et al., 2014). Therefore, our data show that H2A.X monoubiquitylation at K118/119 is not required for H2A.X phosphorylation on S139 *in cis*; however, data from other studies suggests that H2A.X/H2A monoubiquitylation at K118/119

could still be required for H2A.X phosphorylation *in trans*. It should be noted that this hypothesis is applicable to DNA DSB – inducing agents that cause increases in monoubiquitylation of H2A.X at K118/119, such as IR and etoposide. The relationship between monoubiquitylation at K118/119 and phosphorylation at S139 in response to MMS remains unclear, since MMS decreases monoubiquitylation while still being able to increase phosphorylation. Monoubiquitylation at K118/119 may not be important for MMS – induced phosphorylation at S139, but the decrease in monoubiquitylation at this site may have another role in DSB signaling and repair in response to MMS-induced damage.

Our data also showed that H2A.X monoubiquitylation at K118/119 is not required for H2A.X monoubiquitylation at K13/15 *in cis*, as we saw that H2A.X-K118/119R-Flag-BirA was still able to become monoubiquitylated at K13/15. Data from other studies support this finding: monoubiquitylation at K13/15 was seen on H2A.X K118/119R mutants in experiments conducted by other researchers (Leung et al., 2014; Mattiroli et al., 2012). However, it is still possible that H2A.X/H2A monoubiquitylation at K118/119 is required for H2A.X/H2A monoubiquitylation at K13/15 *in trans*: H2A.X/H2A monoubiquitylation at K118/119 may be required for H2A.X phosphorylation at S139 *in trans*, and H2A.X phosphorylation may be required for H2A.X/H2A monoubiquitylation at K13/15 *in trans*. This hypothesis is applicable to DNA DSB – inducing agents that cause increases in H2A.X/H2A monoubiquitylation at both K118/119 and K13/15 sites, such as IR and etoposide. It wouldn't be applicable for MMS, which decreases monoubiquitylation at K118/119 and K13/15. It was unexpected to see that these two H2A.X monoubiquitylation marks decreased in response to MMS, since MMS was seen to increase polyubiquitylation levels on H2A.X, presumably at the K13/15 site. This

suggests that polyubiquitylation of H2A.X/H2A may be independent from monoubiquitylation at the K118/119 and K13/15 sites.

In our experiments, we were unable to determine whether H2A.X phosphorylation at S139 is required for H2A.X monoubiquitylation at K13/15 *in cis*, since it was difficult to detect monoubiquitylation at K13/15 on WT H2A.X-Flag-BirA for comparison to H2A.X-S139A-Flag-BirA. To our knowledge, there are no other studies that examined the effects of an H2A.X S139A mutation directly on the ubiquitylation of H2A.X/H2A at K13/15, or even the effects of an H2A.X S139A mutation on the recruitment of RNF8 or RNF168 to sites of DSBs. One study showed that an H2A.X S139A mutation resulted in impaired recruitment of 53BP1 to sites of DSBs (Leung et al., 2014): this observation supports the theory that H2A.X phosphorylation at S139 is required for H2A.X/H2A monoubiquitylation at K13/15, which is essential for 53BP1 recruitment. It appears that most of the speculation that H2A.X phosphorylation at S139 is required for H2A.X/H2A monoubiquitylation at K13/15 is built upon individual findings from different studies (studies that show phosphorylated H2A.X recruits MDC1, MDC1 recruits RNF8, and RNF8 helps recruit RNF168 to ubiquitylate H2A.X/H2A at K13/15).

By examining the effects of RNF168 overexpression on the ubiquitylation of the various H2A.X-Flag-BirA mutants, we were able to verify that RNF168 is only able to ubiquitylate H2A.X on K13/15, but not K118/119 or any other sites on H2A.X. These observations are consistent with findings from another study (Gatti et al., 2012), which showed similar results. Interestingly, we saw that RNF168 overexpression induced higher levels of monoubiquitylation at K13/15 on H2A.X-K118/119R-Flag-BirA compared to WT H2A.X-Flag-BirA. We ruled out the possibility that RNF168 is able to interact better with nucleosomes that do not contain monoubiquitylated H2A.X-Flag-BirA at K118/119, since we saw similar amounts of RNF168

bound to nucleosomes containing WT H2A.X-Flag-BirA and H2A.X-K118/119R-Flag-BirA. We also saw that RNF168 overexpression was able to increase monoubiquitylation at K13/15 on H2A.X-S139A-Flag-BirA, similarly to WT H2A.X-Flag-BirA. We cannot draw any conclusions about the physiological importance of H2A.X S139 phosphorylation to RNF168 ubiquitylation at K13/15, since overexpressing RNF168 may substitute the requirement for H2A.X phosphorylation to recruit RNF168 to H2A.X. However, we can conclude that H2A.X phosphorylation at S139 is not biochemically required for H2A.X monoubiquitylation at K13/15 by RNF168 *in cis*.

4.3 Concluding Remarks

The goal of this project was to elucidate the mechanisms surrounding H2A.X monoubiquitylation marks in response to DNA DSB formation. Based on findings from the literature, we initially believed that the mechanisms associated with DSB signaling were uniform for all DSBs, regardless of their sources. However, after testing different DSB – inducing agents, we have seen that different DSB – inducing agents can have different effects on H2A.X ubiquitylation. This finding is significant in that it shows that there may be different signaling mechanisms associated with different types of DSB – inducing agents. Although we only examined the effects of exogenous DSB – inducing agents like MMS and etoposide, it is possible that different endogenous sources of DSBs can have different signaling mechanisms as well.

We saw that MMS decreases H2A.X monoubiquitylation at K118/119 and at K13/15, whereas etoposide increases monoubiquitylation on H2A.X at K118/119 and K13/15. We were able to determine that H2A.X monoubiquitylation at K118/119 is not required for H2A.X

phosphorylation at S139 or H2A.X monoubiquitylation at K13/15 *in cis*. Additionally, our results suggest that monoubiquitylation at K118/119 may have an antagonistic effect on monoubiquitylation at K13/15. Using our novel purification approach for the isolation of biotinylated/ubiquitylated H2A.X – containing nucleosomes, we have the potential of isolating monoubiquitylated (at K118/119) H2A.X – containing nucleosomes in response to MMS and etoposide. This gives us the opportunity to identify proteins that are associated with monoubiquitylated (at K118/119) H2A.X – containing nucleosomes in response to these two DNA damage – inducing agents. It would be advantageous to gain more knowledge of the mechanisms associated with DSB signaling and repair in response to MMS and etoposide, especially for etoposide since it is used in the treatment of cancers. Additionally, we also have the potential of discovering other nucleosome-associated proteins that become ubiquitylated in response to DSBs induced by MMS, etoposide and doxorubicin. Overall, our findings would provide more insight into the diverse array of mechanisms associated with DNA DSB signaling.

CHAPTER 5: FUTURE EXPERIMENTS

Before using the streptavidin-coupled beads again for subsequent experiments, we would need to find ways to remove the non-specific binding of proteins to streptavidin-coupled beads as shown in **Figure 21A**. We could try incubating lysates with biotin-saturated streptavidin-coupled beads to remove proteins that bind the beads non-specifically (i.e. non biotin-dependent manner). Such a method of pre-clearing lysates before actual IP/pull-down is often used in affinity purification procedures. Recently, another lab member used streptavidin-coupled beads from a different source (agarose beads instead of the sepharose ones used in my experiments), and found significantly less non-specific protein binding with the agarose beads. Therefore, it would be worthwhile testing and comparing the two types of streptavidin-coupled beads with my pull-down schemes to see if that would help reduce the non-specific binding problem. Finally, we could also do a tandem purification instead, whereby we would immunoprecipiatate all H2A.X-Flag-BirA proteins with anti-Flag antibodies, and then subject the immunoprecipitated proteins to pull-downs with streptavidin-coupled beads. The beads used for Flag IPs have very low levels of non-specific protein binding, so the majority of non-specific proteins should be eliminated at that stage.

Once we could eliminate the non-specific protein binding problem, we would repeat our previously conducted experiment, where we would pull down monoubiquitylated (at K118/119) H2A.X-Flag-BirA – containing nucleosomes under normal, MMS-treated, and etoposide-treated conditions. With the removal of all the non-specific proteins, hopefully we would be able to see more differences in eluted proteins among the three samples. To determine the identity of a particular protein, such as the 20 kDa one we saw interact with nucleosomes in response to MMS and etoposide, we could cut out the protein band from the gel and prepare for it to be sent for

mass spectrometry analysis. Alternatively, we could send all the eluted proteins from each sample for mass spectrometry analyses, determine the identities and relative abundances of all the eluted proteins from each sample, and then compare them.

Additionally, we would conduct an experiment to determine the identities of the other biotinylated/nucleosome-associated proteins that become ubiquitylated in response to MMS, etoposide and doxorubicin. We would co-express H2A.X-Flag-BirA with Avi-tagged ubiquitin in cells, and treat cells with sterile water or MMS. Nuclear lysates would be prepared, and proteins would be denatured to abolish protein-protein interactions. The nuclear lysates would then be subjected to pull-downs with streptavidin-coupled beads. Biotinylated proteins would be eluted, and the identities and relative abundances of proteins in both samples would be determined using mass spectrometry.

We have seen that the majority of H2A.X-Flag-BirA – containing nucleosomes that are isolated with streptavidin-coupled beads are monoubiquitylated on H2A.X at K118/119. Therefore, it is difficult to isolate H2A.X-Flag-BirA – containing nucleosomes that are monoubiquitylated on H2A.X at K13/15. To enrich for H2A.X-Flag-BirA – containing nucleosomes that are monoubiquitylated on H2A.X at K13/15, we could use the H2A.X-K118/119R-Flag-BirA mutant: we would be able to isolate more nucleosomes that are monoubiquitylated on H2A.X-Flag-BirA at K13/15 by eliminating nucleosomes that are monoubiquitylated on H2A.X-Flag-BirA at K118/119. RNF168 overexpression and etoposide treatment could be used in combination to increase levels of monoubiquitylation on H2A.X-K118/119R-Flag-BirA at K13/15. Therefore, we would co-express H2A.X-K118/119R-Flag-BirA with Avi-tagged ubiquitin and HA-RNF168 in cells, and then treat cells with etoposide. We would also co-express H2A.X-Flag-BirA with Avi-tagged ubiquitin and an empty pBABE

vector, and then treat cells with DMSO: this sample would be used for comparison, since the majority of H2A.X-Flag-BirA – containing nucleosomes isolated with streptavidin-coupled beads from this sample should be monoubiquitylated on H2A.X at K118/119 for transcriptional repression. After nucleosomes from both samples are subjected to pull-downs with streptavidin-coupled beads, the identities of eluted proteins from both samples could be determined using mass spectrometry. A comparison of proteins in both samples would give us the opportunity to discover proteins that interact with nucleosomes in response to monoubiquitylation of H2A.X at K13/15.

In our previous experiments, we were unable to gain a better understanding of the relationship between H2A.X phosphorylation at S139 and H2A.X monoubiquitylation at K13/15. This was because it was difficult to detect monoubiquitylation at K13/15 on WT H2A.X-Flag-BirA for comparison to H2A.X-S139A-Flag-BirA. Since monoubiquitylation at K13/15 could clearly be seen on H2A.X-K118/119R-Flag-BirA, it would be useful to make an H2A.X-Flag-BirA construct that has both K118/119R and S139A mutations. H2A.X-K118/119R-Flag-BirA and H2A.X-K118/119R, S139A-Flag-BirA would be individually expressed in 293T cells, which would subsequently be treated with etoposide. A comparison of monoubiquitylation levels at K13/15 between these two proteins would indicate whether H2A.X phosphorylation at S139 is required for H2A.X monoubiquitylation at K13/15 *in cis*. If it is not required *in cis*, we could test to see if it is required *in trans* by repeating this experiment using H2A.X$^{-/-}$ cells: if H2A.X-K118/119R, S139A-Flag-BirA is expressed in H2A.X$^{-/-}$ cells, there will be no endogenous H2A.X S139 phosphorylation to potentially support H2A.X monoubiquitylation at K13/15 *in trans*. If we find that H2A.X phosphorylation at S139 is required for H2A.X monoubiquitylation at K13/15 *in trans*, we could test to see if H2A.X phosphorylation is important for the

recruitment of RNF8 and RNF168. WT H2A.X-Flag-BirA and H2A.X-S139A-Flag-BirA could be individually expressed in H2A.$X^{-/-}$ cells, and subsequently treated with etoposide. The H2A.X-Flag-BirA – containing nucleosomes could then be immunoprecipitated with anti-Flag antibodies, and levels of co-purified RNF8 and RNF168 could be compared between the two samples. Although we didn't get the chance to, it could still be useful to examine the recruitment of other DNA DSB – related proteins to nucleosomes containing the other H2A.X-Flag-BirA mutants as well. WT H2A.X-Flag-BirA, H2A.X-K118/119R-Flag-BirA, and H2A.X-K13/15R-Flag-BirA could be individually expressed in H2A.$X^{-/-}$ cells, and subsequently treated with etoposide. The H2A.X-Flag-BirA – containing nucleosomes could then be immunoprecipitated with anti-Flag antibodies, and levels of co-purified proteins like 53BP1 could be compared among the samples. This could help us gain a better understanding of the mechanisms surrounding H2A.X monoubiquitylation at K118/119 and at K13/15.

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