Nuclear Arp2/3 drives DNA double-strand break clustering for homology-directed repair

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Severing the DNA double helix is a requisite step in the exchange of genetic material between homologous chromosomes in meiosis and between immunoglobulin domains during the generation of immune-receptor diversity. While these DNA transactions are essential for human fertility and the development of the immune system, misrepaired or unrepaired DNA double-strand breaks (DSBs) can lead to chromosome rearrangements or cell death. Indeed, ionizing radiation which generates DSBs in tumors is a cornerstone of cancer therapy. However, tumor cells can tolerate otherwise lethal levels of DNA damage by exploiting DNA repair pathways. Thus, discovering new strategies to selectively inhibit the repair of DSBs remains a major goal in the development of more effective cancer therapies.

DSB repair may occur by multiple pathways, and the decision to use one pathway over another is influenced by cell cycle stage, the chromatin state, and the complexity of the inciting lesion. Mammalian cells primarily resolve DSBs by ligating the free ends together during a process termed “non-homologous end joining” (NHEJ). However, chemically modified or damaged DSB ends cannot be directly ligated by the NHEJ machinery. If NHEJ fails, DSBs may be nuclease-cleaved to generate 3’ single-stranded DNA overhangs via a process called end resection. The resected DNA strands are poor substrates for NHEJ and instead search for homology in the genome to resynthesize the sequence surrounding the break site. This process is termed “homology-directed repair” (HDR). HDR is tightly coupled to cell cycle phase to ensure
that resection occurs during late S and G2 when the ideal template, the sister chromatid, may be utilized.

Following DNA damage, repair factors accumulate at DSB sites and form microscopically-detectable DNA repair foci. The dynamics of these foci may be observed by time-lapse microscopy making it possible to observe the behavior of breaks undergoing HDR and NHEJ. Interestingly, in yeast and mammalian cells, DNA motion is increased following DSB generation. DNA movements can lead to the clustering of DSBs into a common repair focus. DSB movements are intricately related to repair by HDR and require factors critical for resection initiation and downstream recombination. In contrast, DSBs undergoing NHEJ are relatively immobile. These observations suggest that the commitment of DSB repair to HDR regulates DSB movement and clustering; however, how DSB clustering might promote repair and whether active mechanisms drive this process remain relatively obscure.

Recent studies have proposed roles for cytoskeletal proteins in genome organization and chromosomal dynamics. The Arp2/3 complex generates propulsive forces by nucleating a highly branched network of actin filaments. Genotoxic agents trigger actin polymerization in the nucleus. However, how DSB repair pathways might harness nuclear Arp2/3 machinery is unknown. Chapter 1 provides an overview of these pathways including the key steps of DSB repair, the regulation of actin nucleation, and the proteins involved in chromatin mobility. Chapter 1 provides context for the rest of the thesis in which I explore the contribution of nuclear actin polymerization to DSB repair.

In Chapter 2, I detail our studies assessing the contribution of the Arp2/3 complex to DSB movement and clustering. Using *Xenopus laevis* cell-free extracts and mammalian cells, we show that actin nucleation machinery (WASP, Arp2/3, and actin) is recruited to damaged chromatin undergoing HDR. In this chapter, I also investigate how Arp2/3-driven DSB movements specifically promote the dynamics of HDR breaks, while
Arp2/3 activity does not influence NHEJ breaks. Finally, I show that reduced DSB movement produces defects in DNA end processing and HDR efficiency, while the efficiency of end-joining is unaffected.

I summarize all of these findings in Chapter 3 and discuss their implications for DNA repair, translocation formation, and clinical applications.
# Table of Contents

List of Figures and Tables........................................................................................................ iv
Acknowledgements .................................................................................................................. vi
Dedication............................................................................................................................... viii

**Chapter 1. Introduction**...................................................................................................... 1

1. **PRINCIPLES OF GENOME ORGANIZATION**................................................................. 2

   a. A historical perspective............................................................................................... 2

   b. Constraints to chromatin movement......................................................................... 9

2. **DNA DOUBLE-STRAND BREAKS REORGANIZE CHROMATIN**................................. 15

   a. DNA lesions and their repair pathways................................................................. 15

      i. General principles............................................................................................. 14

      ii. Double-strand breaks and their repair....................................................... 17

   b. DNA double-strand breaks move...................................................................... 27

      i. Fluorescently-tagged proteins reveal DSB dynamics............................. 27

      ii. Assessing chromatin mobility: MSDs, D(t)s and α’s.................... 28

      iii. DSB movement and repair pathway choice................................. 32

   c. The genetic requirements for chromatin movement................................. 38

      i. DSB repair machinery................................................................................. 38

      ii. Nucleoskeletal anchors and cytoskeletal forces................................ 39

      iii. Chromatin remodelers............................................................................ 41

   d. Types of DSB movement............................................................................... 44

      i. Homology search.......................................................................................... 44

      ii. DSB clustering......................................................................................... 44
iii. DSB relocalization................................................................. 45
e. Translocations: an undesired consequence of DSB motion?...... 47

3. A ROLE FOR POLYMERIZED ACTIN IN DNA TRANSACTIONS........ 47
   a. Actin nucleators and nucleation promoting factors............... 49
      i. WASP and Arp2/3.............................................................. 49
      ii. Regulation of WASP-Arp2/3 dynamics................................. 56
      iii. The Formin family......................................................... 57
   b. Actin monomers and polymers drive nuclear processes........... 57
      i. DNA repair........................................................................ 59
      ii. Chromatin remodeling..................................................... 60
      iii. Chromatin organization and decompaction....................... 61
      iv. Transcription..................................................................... 62

4. WASP AND ARP2/3 IN HEALTH AND DISEASE.......................... 66
   a. Wiskott-Aldrich Syndrome................................................... 66
   b. X-linked neutropenia.......................................................... 68

Chapter 2. Manuscript........................................................................ 69
   1. Author Contributions............................................................ 70
   2. Abstract................................................................................ 70
   3. Introduction............................................................................ 72
   4. Results.................................................................................. 73
   5. Discussion............................................................................. 132
   6. Methods............................................................................... 133

Chapter 3. Conclusion....................................................................... 151
1. Discussion and Future Directions............................................................... 152
2. Concluding remarks.................................................................................. 168

References........................................................................................................ 169
List of Figures and Tables

Chapter 1:
1-1: A historical perspective of genome organization.................................................. 6
1-2: Chromatin organization in mammalian cells and yeast......................................... 13
1-3: Non-homologous end joining (NHEJ) and homology-directed repair (HDR)
pathways compete for DNA double-strand breaks (DSBs)........................................... 25
1-4: Types of particle movement..................................................................................... 30
1-5: Damaged chromatin moves...................................................................................... 36
1-6: Genetic Requirements for DSB movement............................................................... 42
1-7: Actin polymerization by WASP, Arp2/3, and related proteins................................. 54
1-8: Functions for nuclear actin....................................................................................... 64

Chapter 2:
2-1: Actin complexes are recruited to damaged chromatin............................................ 75
2-2: Actin filament nucleators localize to chromosomal DSBs in Xenopus extracts
and mammalian cells....................................................................................................... 77
2-3: Source gels................................................................................................................ 79
2-4: Arp2/3 and WASP co-localize at HDR breaks......................................................... 83
2-5: Arp2/3 drives DSB mobility during HDR.................................................................. 91
2-6: CK-689 does not significantly alter movement of foci............................................ 93
2-7: Arp2/3 clusters Rad51 foci......................................................................................... 95
2-8: WASP and Arp/3 inactivation do not impair U2OS cell viability, nuclear area,
nuclear sphericity or protein expression.......................................................................... 97
2-9: Arp2/3 enhances movement of 53BP1 foci in G2 cells................................. 99
2-10: Arp2/3 clusters DSBs with slow kinetics of repair.................................... 101
2-11: WASP and Arp2/3 cluster γH2AX foci in U2OS and MEFs......................... 104
2-12: Nuclear actin foci cluster and localize to HDR sites................................ 108
2-13: Arp2/3 promotes actin foci assembly following DNA damage.................. 110
2-14: GFP-based reporter assays for DSB repair............................................... 114
2-15: WASP and Arp2/3 mediate HDR.............................................................. 116
2-16: WASP and Arp2/3 mediate DSB repair by homology-directed mechanisms. .......................................................... 118
2-17: Actin nucleation regulates HDR in the nucleus and does not require formin-2 activity......................................................................................... 120
2-18: Arp2/3 facilitates resection and repair in G2............................................. 125
2-19: B-lymphocytes derived from Wiskott-Aldrich Syndrome patients exhibit reduced DSB end-resection................................................................. 128
2-20: Arp2/3 inactivation confers sensitivity to DSBs induced in S-phase as well as replication stress-inducing agents......................................................... 130

Chapter 3:

3-1: Arp2/3 polymerizes actin during HDR to drive foci clustering.................. 153
3-2: Clustering facilitates extensive DSB end-resection.................................... 158
3-3: DSB clustering may promote or prevent chromosomal translocations...... 162
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Dedication

This thesis is dedicated to my partner Andrew Hollar. Coming home to you is the best part of my day. I wish everyone could be so lucky.
Chapter 1. Introduction
1. PRINCIPLES OF GENOME ORGANIZATION

A. A historical perspective

For seventy years before the discovery of DNA, genetics was defined by studies of chromosomal dynamics. Careful observation of mitotic cells revealed chromosomes that dramatically rearranged prior to cytokinesis. Whether chromosomes occupied defined nuclear positions in interphase cells was investigated and debated. Following the discovery of DNA, however, the study of nuclear architecture was abandoned as efforts to understand how genes were encoded intensified. This endeavor culminated in the sequencing of the human genome in 2001. Since then it has become evident that the DNA nucleotide sequence provides but a one-dimensional understanding of genomic function. Thus, efforts to understand central DNA transactions like transcription, replication, and repair have refocused on studies of nuclear architecture.

Early 19th century thinkers postulated that genomes were generated de novo by a mixture of nutrients, minerals, and elements (Cremer and Cremer, 2006). The first evidence that genetic material might be passed from generation to generation arose from the work of Walther Flemming in 1882 (Flemming, 1965). Using salamander embryos stained with aniline dyes, Flemming observed long threads within the nucleus that assembled at the equatorial plate, split longitudinally, and regressed as the cells passed through mitosis (Fig. 1-1a). Ultimately, the threads became the genetic content of daughter cells, evidence that provided the basis for Mendel’s theory of inheritance proposed years prior.
The first insight that chromosomes might occupy distinct nuclear territories stemmed from Theodor Boveri’s studies of the horse roundworm *Ascaris megaloecephala*. In 1888, Boveri observed small protrusions extending from the nuclear membrane in *Ascaris* which correlated with the location of chromosome ends. The protrusion pattern remained relatively constant throughout interphase indicating that chromosomes occupied positions that were relatively stable (Fig. 1-1b). Moreover, while daughter cells shared nearly symmetrical patterns of nuclear protrusions, the patterns shifted in subsequent mitoses. This suggested that chromosome positioning was fixed during interphase yet not heritable. Boveri imagined that nuclei were divided into distinct territories separated by interchromosomal space (Fig. 1-1c). Similarly, Eduard Strasburger claimed that nuclear territories were comprised of clumps of chromatin in plants in 1905 (Fig. 1-1d).

Nearly eighty years later, Thomas and his wife Marion Cremer garnered the first experimental evidence that showed that interphase nuclei possess a high degree of spatial organization. Using a UV laser, the team microirradiated discrete locations within Chinese hamster nuclei (Cremer et al., 1982). By pulsing cells with 3H-thymidine, they detected DNA photolesions that underwent base excision repair by autoradiography. Incredibly, microirradiation marked only a subset of chromosomes indicating that they occupied distinct territories (Fig. 1-1e). Upon the advent of chromosome painting (Cremer and Cremer, 2001), Boveri’s theory of nuclear organization was finally accepted.
Today, it is recognized that mammalian chromosomes dwell within 1-2 µm territories. While no two cells exhibit identical chromosomal topology, small, gene-dense chromosomes tend to congregate in the center of the nucleus while gene-poor chromosomes localize to the nuclear periphery (Croft et al., 1999; Fig. 1-1f, left). At the resolution of megabases, gene-rich euchromatin is spatially segregated from gene-poor heterochromatin. At kilobase resolution, chromosome territories can be further resolved into topologically associated domains (TADs). TADs are defined by high-throughput chromosome capture (Hi-C) technology as preferentially clustered genomic loci (Dekker et al., 2013). TADs maintain epigenetic control over gene expression by facilitating long-range interactions between promoter and enhancer regions (Fig. 1-1f, right). TADs may also serve as regulatory compartments for DNA synthesis, as those with high transcriptional activity replicate first followed by later-replicating heterochromatic TADs (Pope et al., 2014). In the budding yeast Saccharomyces cerevisiae recent Hi-C data indicate that chromosomes also harbor TADs that segregate early from late regions of DNA replication (Eser et al., 2017). This suggests that genome compartmentalization into TADs may provide a unified model for chromosome structure and function in eukaryotes.

At base pair resolution, nucleosomes comprise the basic units of chromatin. Formed by histone octamers around which DNA molecules turn, nucleosomes occupy distinct positions along chromosomes, and can trigger changes to chromatin behavior upon their phosphorylation, methylation, or acetylation (Badeaux and Shi, 2013). For example, acetylation of lysine residues
on histones weakens their interaction with DNA thereby granting access to transcription machinery. Indeed, H3 acetylation on K9 marks active gene promoters and is thought to mediate transcription elongation by Pol II (Gates et al., 2017). In contrast, trimethylation of H3 K9 is thought to induce a more compact nucleosome structure thereby silencing chromatin (Choy et al., 2010; Hyun et al., 2017). Thus, the positioning of chromosomal territories, the interaction of loci within TADs, and the post-translational modification of nucleosomes together define the hierarchical organization of DNA.
Fig. 1-1 | A historical perspective of genome organization.
Fig. 1-1 | A historical perspective of genome organization. a) Drawings of mitotic structures in salamander embryos originally discovered by Walther Flemming in 1882. Flemming observed nuclear threads that longitudinally split at the equatorial plate and regressed to opposing poles of the cell. These pieces of thread subsequently formed the nuclear substance of the daughter nuclei. Adapted from Flemming, 1965. b) Drawings of chromosomes in prophase nuclei of the horse roundworm by Theodor Boveri. Boveri noticed that chromosome ends abutted the nuclear membrane forming small protuberances (arrows). Nuclei with similar patterns of nuclear protrusions (compare top vs. bottom) are daughter nuclei that originated from a previous mitosis. Nuclear protrusions in daughter nuclei remained relatively fixed throughout interphase indicating that chromosome territories are positionally stable. Adapted from Cremer and Cremer, 2010. c) A speculative drawing of chromosome territories by Boveri in 1888. The drawing shows two intermingling chromosome territories which contain neighboring strands of chromatin separated by interchromatin space. Adapted from Cremer and Cremer, 2006. d) A drawing of a plant cell nucleus from Galtonia candicans by Eduard Strasburger in 1905. Blue and red spots represent chromatin foci which comprise the chromosome territories. Adapted from Cremer and Cremer, 2006. e) Representative image of a Chinese hamster nucleus (left) or metaphase spreads (right) following laser-UV-microirradiation by Cremer and colleagues in 1982. DNA photolesions were detected by 3H-thymidine pulse-labeling which reflects DNA synthesis during base excision repair. Microirradiation of a small nuclear territory (large arrow) marks a subset of
chromosomes (small arrows, 1 and 2, right). This supports the idea that chromosomes are organized into discrete, rather than diffuse, chromosomal territories. Adapted from Cremer et al., 1982. f) A recent model of nuclear organization in mammals adapted from Gibcus and Dekker, 2013. Chromosome painting reveals the arrangement of chromosomes within restricted sub-nuclear zones (Green, Chromosome 18; Red, Chromosome 3). At the resolution of several hundred kilobases, chromosomal territories are comprised of topologically associating domains (TADs, gray circles) which reflect the preferential interaction of enhancer and promoter genomic elements. TADs congregate to form compartments (A & B) which reflect their genomic content and chromatin status.
B. Constraints to chromatin movement

While promoters and enhancers intermingle within TADs, chromosomal territories remain spatially discrete. This occurs partially by virtue of nuclear crowding as chromosomes abut one another inside a space with limited volume. However, genomic loci associated with the nuclear periphery exhibit more constrained motion relative to those within the nucleoplasm (Chubb et al., 2002). In mammalian cells, this confinement is likely stabilized by chromosomal interactions with the nuclear lamina.

The nuclear lamina is comprised of a meshwork of intermediate filaments called Lamins which polymerize inside the nuclear envelope. Large stretches of heterochromatin contain lamin-associated domains (LADs) which bind proteins embedded in the lamina. These interactions are mediated in part by the lamin-B receptor which binds core heterochromatic components HP1α and H4K20me3 (van Steensel and Belmont, 2017). Silencing of lamin B1 results in the redistribution of repressed genes from the nuclear periphery towards the interior (Camps et al., 2014). On the other hand, lamin A is present not only around the nuclear periphery but also throughout the nucleoplasm. Lamin A colocalizes at euchromatic gene regions with the chromatin-binding protein lamin-associated polypeptide (LAP) 2 alpha (Gesson et al., 2016). In contrast to lamin B, loss of lamin A increases the motion of telomeres located at the periphery as well as those more internal (Bronshtein et al., 2015). This suggests that lamin A may crosslink chromatin to globally constrain motion.
Heterochromatin is also tethered to the nucleolus via nucleolus-associated domains (NADs) which share substantial sequence overlap with LADs. In fact, heterochromatic LADs that do not associate with the nuclear lamina wrap around the nucleolus after mitosis (Kind et al., 2013). Moreover, disruption of nucleolar structure enhances the movement of associated genomic loci (Chubb et al., 2002).

The higher-order chromatin architecture established by LADs and NADs is further cemented at the kilobase level by the CCCTC-binding factor CTCF and structural maintenance of chromosome (SMC) complexes. CTCF localizes to chromatin loops that facilitate communication between genes and regulatory domains within TADs (Splinter et al., 2006). Loss of CTCF blurs the boundaries between adjacent TADs suggesting that CTCF specifies loop positioning (Wutz et al., 2017). In contrast, the SMC complex cohesin is thought to bind DNA sequences in cis to generate loops (Wendt et al., 2008). Accordingly, loss of the cohesin-loading factor Nipbl completely abolishes chromosomal loops and leads to the disappearance of TADs (Schwarzer et al., 2017). Similarly, condensin II knockdown increases the frequency of interchromosomal mixing in Drosophila (Rosin et al., 2018). Taken together, these observations suggest that CTCF and SMC complexes work together to establish chromatin loops within TADs.

In contrast to mammalian cells, yeast lack a nuclear lamina and rely upon alternative measures to establish nuclear organization. The genome of the budding yeast Saccharomyces cerevisiae contains 16 chromosomes which are arranged in a highly polarized manner (Lorenz et al., 2002). The spindle pole
body (SPB) serves as the microtubule organizing center and lies directly opposite the nucleolus which abuts the nuclear envelope (Fig. 1-2b). Heterochromatic centromeres cluster around the SPB in a rosette conformation forming a distinct subnuclear compartment that anchors chromosomes. This organization constrains chromatin mobility, and indeed loss of centromere tethering increases chromosome movement (Verdaasdonk et al., 2013). From the centromere chromosome arms extend outward; telomeres and subtelomeres cluster at the nuclear periphery via interactions with proteins embedded in the nuclear membrane. These interactions are mediated in part by the Yku70/ku80 complex and Sir4 which anchor telomeres to Esc1 on inner face of the nuclear envelope (Taddei et al., 2004). Accordingly, deletion of Yku70 or Sir4 disrupts telomere tethering at the periphery (Bystricky et al., 2005).

Despite the fact that chromosomal tethering at the SPB and nuclear envelope establishes polarity, nuclear organization in yeast is hardly static. Budding yeast chromosomes have higher diffusion coefficients than human chromosomes indicating less confinement to motion (Marshall et al., 1997; Chubb et al., 2002). Indeed, many features of the S. cerevisiae genome such as gene territory topology, chromosomal folding patterns, and intra/interchromosomal contacts can be explained by random chromosome intermingling (Tjong et al., 2012). Thus, unlike mammalian cells, chromosomal tethering to the nuclear periphery dictates the spatial positioning of only a subset of genes near telomere ends (Avsaroglu et al., 2014). Consistent with this notion,
genomic loci distal to yeast centromeres are 10-fold less confined than those at more proximate locales (Heun et al., 2001).

Finally, in yeast and mammalian cells, chromosome movements may become more confined by replication and transcriptional transactions. Following DNA replication, chromatin motion is reduced suggesting that cohesin loading or increased nucleoplasm viscosity may further constrain motion. Furthermore, transcriptional machinery deposits the SMC complex condensin on tRNA genes which organizes nucleolar architecture (Haeusler et al., 2008).
Fig. 1-2 | Chromatin organization in mammalian cells and yeast.
Fig. 1-2 | Chromatin organization in mammalian cells and yeast. a) Heterochromatin (HC) is tethered to lamin B (LMN B) at the nuclear periphery via the lamin B receptor which associates with HP1α (red stars, upper left box). In contrast, euchromatin (EU) is anchored to lamin A (LMN A) via lap2α (upper box, right). Topologically associated domains (TADs) are also held in close proximity by structural maintenance of chromosome (SMC) complexes like cohesin. Finally, heterochromatin domains that do not associate with the nuclear periphery anchor to the nucleolus via nucleolar associated domains (NADs) (lower box, right). b) In budding yeast, chromosomes are tethered at the centromere which lies opposite the nucleolus. Telomeres are anchored to the nuclear periphery via interactions between ku70/ku80 or Sir4 and Esc1. In contrast to mammalian chromosomes, intra and interchromosomal contacts may be predicted by random-walk polymer modeling (inset, right).
2. DNA DOUBLE-STRAND BREAKS REORGANIZE CHROMATIN

The hierarchal three-dimensional organization of the nucleus is preserved by loops that maintain intrachromosomal contacts and tethers that spatially segregate chromosomal compartments. However, when cells suffer DNA damage chromatin reorganizes to preserve genome integrity.

A. DNA lesions and their repair pathways

i. General principles

Perturbations to genome integrity arise during a variety of endogenous processes. For example, during replication DNA Polymerase α erroneously incorporates 1 out of every 10,000 nucleotide bases (Arana and Kunkel, 2010). DNA lesions also arise from a myriad of exogenous sources, the most pervasive of which is the sun. A cell exposed to one hour of intense sunlight can suffer damage to 100,000 sites in the genome (Jackson and Bartek, 2009). Regardless of their etiology, DNA lesions are rapidly healed by a process collectively termed the DNA damage response (DDR) (Ciccia and Elledge, 2010). Speaking broadly, the DDR achieves three goals: 1.) DNA damage recognition by proteins that surveil the genome; 2.) Processing of the damaged site; and 3.) Translesion DNA synthesis that restores genome integrity. This framework allows the cell to restore heterogeneous DNA insults ranging from those that minimally damage DNA bases (e.g. deamination and oxidation) to those that distort the double-
stranded conformation of the helix (e.g. interstrand crosslinks, ICLs) to those that sever the helix itself (i.e. double-strand breaks, DSBs).

The machinery that carries out the DDR is often shared by pathways that repair distinct lesions. For example, following ICL unhooking, the DNA strand bearing the crosslinked adduct is trimmed by the exonuclease Exo1 in replication independent repair (Kato et al., 2017). In contrast, Exo1 is also recruited to sites of DNA end resection during homology-directed repair as will be discussed below. Similarly, chromatin remodeling complexes localize to sites of UV irradiation where they mediate the chromatin elongation and decondensation (Hittelman and Pollard, 1984; Farrell et al., 2011). Chromatin remodelers also promote the sliding and eviction of nucleosomes to increase the accessibility of DSB sites to repair factors.

Interestingly, despite their shared machineries, certain properties of damaged DNA remain distinct. For example, the mobility of damaged DNA sites varies greatly by the type of DNA damage induced and the repair pathway activated. As will be discussed below, cells damaged by localized UV laser microirradiation do not exhibit increased chromatin motion relative to undamaged cells (Kruhlak et al., 2006). In contrast cells that suffer DNA double-strand breaks (DSBs) display a dramatic increase in chromosome mobility. This suggests that DSBs recruit distinct machinery that alters nuclear architecture.
ii. **Double-strand breaks and their repair**

Although much less common than ultraviolet light, exposure to ionizing radiation can sever the DNA double helix. DNA DSBs are lethal to eukaryotic cells as evidenced by the 49 immediate fatalities following the 1986 Chernobyl disaster which released 10,000 petabecquerel of radionucleotides into the atmosphere (Moller and Mousseau, 2015). Moreover, DSBs promote chromosomal translocations that favor oncogenesis, and indeed 6,000 cases of thyroid cancer developed in the years following the same incident (Charles, 2001). Today, exposure to natural or man-made radioisotopes is commonly utilized in clinics to increase the DSB burden in cervical cancers, breast cancer, Hodgkin lymphoma, and testicular cancer, among others.

Human cells generate as many as twenty DSBs per day as a consequence of endogenous sources of DNA damage. However, DSBs are also programmed to emerge during processes essential for life. In the prophase prior to meiosis I, DSBs are generated by the topoisomerase-II-related enzyme Spo11. These DSBs enable the exchange of genetic information between homologous chromosomes which is required for chromosome segregation (Murakami and Keeney, 2008). Hence DSBs are essential for the development of genetically diverse gametes. Similarly, diverse repertoires of immune receptors are generated by DSBs between V, D, and J segments of antigen-binding genes. Unique V, D, J combinations allow developing B and T lymphocytes to produce immunoglobulin and T-cell receptors that detect a range of pathogens.
DSB repair is carried out by multiple pathways. The decision to use one pathway over another is influenced by cell cycle stage, the chromatin state, and the complexity of the lesion itself. DSBs are recognized within seconds of their appearance by PARP1 which catalyzes the addition of negatively charged poly(ADP-ribose) (PAR) chains to histones around the damaged site (Schreiber et al., 2006; Yang et al., 2018). PAR polymers promote the assembly of intrinsically disordered proteins (IDPs) at DSBs (Altmeyer et al., 2015). These IDPs, like FUS/TLS (fused in sarcoma/translocated in sarcoma), contain positively charged low complexity repeats (e.g. RGG) that promote aggregation (Mastrocola et al., 2013). Thus, electrostatic interactions between PAR and IDP moieties are thought to form liquid compartments by phase separation that permits the recruitment and retention of downstream repair proteins (Kai, 2016).

Following PARylation of the DSB, the MRN/X complex localizes to the DNA lesion. MRN/X contains the nuclease Mre11 and its invariant cofactor Rad50 which has SMC-like core domains. Dimerized MRN/X is thought to sandwich the dsDNA molecule between the catalytic head domains of Rad50 (Liu et al., 2016). ATP hydrolysis at the Walker A/Walker B domains on Rad50 unwinds the DNA helix and exposes it to Mre11’s nuclease active site (Liu et al., 2016; Seifert et al., 2016). Mre11 then acts as an endo- and exo-nuclease as will be discussed in detail below.

The DNA processing activity of Mre11 is modulated by the third component of the complex: Nbs1 (expressed in mammals and fission yeast). In contrast, the homolog of Nbs1 in budding yeast, Xrs2, provides the nuclear
localization sequence for the MRX complex but does not regulate Mre11-dependent DSB processing (Oh et al., 2016). However, both Nbs1 and Xrs2 serve the essential role of recruiting the PI3K-like kinase (PIKK) ATM/Tel1 to the DSB site. ATM has hundreds of substrates involved in the DNA damage response including the specialized histone variant H2AX (Matsuoka et al., 2007). H2AX phosphorylation on Ser139 (called γH2AX) spreads for megabases around the DSB site and is thought to promote the retention and downstream ubiquitination of repair factors (Rogakou et al., 1999).

Following ATM activation, two main repair pathways mend DSBs. Non-homologous end joining (NHEJ), which promotes rapid ligation of DSB ends, is the primary repair pathway employed by mammalian cells. NHEJ is initiated when the Ku70/Ku80 heterodimer loads onto to DSB ends and recruits the catalytic subunit of DNA-PK (DNA-PKcs) (Fig. 1-3a). DNA-PK, another PIKK, autophosphorylates on its ABCDE cluster which allows the nuclease ARTEMIS to trim 1- to 5- nucleotide overhangs around the DSB site (Chang and Lieber, 2016). This generates DSBs with compatible ends that may be rejoined by ligation machinery. Notably, budding yeast lack DNA-PK and ARTEMIS homologs which restricts NHEJ to blunt-ended and cohesive DSBs (Dudasova et al., 2004). Subsequent autophosphorylation of DNA-PK on the PQR cluster shields DSB ends from additional end processing and commits repair to NHEJ (Neal et al., 2014). End ligation is carried out by DNA ligase IV (Dnl4/Lig4 in yeast) and accessory proteins Xrcc4 and Xlf (Lif1 and Nej1 in yeast, respectively). While inhibited during mitosis, NHEJ is active throughout
interphase and thus provides an efficient means to repair DSBs without need for a homologous template.

DSB lesions vary by complexity. Those that are chemically modified or blocked by bulky adducts cannot be ligated by NHEJ machinery (Aparicio et al. 2016; Shibata et al., 2011). Instead, these DSBs are processed to generate a 3’ DNA overhang via a process called DNA end resection (Fig. 1-3b). In S. cerevisiae, resection of DSBs with complex ends begins when the MRX complex induces a nick in the 5’ stand via the endonucleolytic activity of Mre11 (Garcia et al., 2011). Oligonucleotides are subsequently exonucleolytically cleaved in a 3’ to 5’ direction towards the break site which generates a short single-stranded DNA overhang.

In yeast and mammalian cells, endonucleolytic cleavage by Mre11 also occurs upstream of DSBs bound by the Ku complex. In this case, Mre11 processing is potentiated by CtIP (Sae2 in budding yeast) which is phosphorylated by cyclin-dependent kinase (CDK) 2 in S and G2-phase cells (Anand et al., 2016; Cannavo and Cejka, 2014, Huertas et al., 2008). By restricting CDK-mediated activation of CtIP to S and G2, resection is coordinated to occur after DNA replication when a sister chromatid is present for repair. Thus, DSB resection and consequent repair by homology-directed mechanisms compensates when Ku is unable to orchestrate the joining of complex ends.

Mre11 endonuclease and exonuclease activities may be abrogated via small molecule inhibitors PFM01 and mirin, respectively (Shibata et al., 2014; Dupre et al., 2008). However, CtIP itself harbors endonuclease activity regulated
by ATM phosphorylation and may resect DSBs when Mre11 is impaired (Arora et al., 2017). Moreover, in contrast to its role in stimulating Mre11 in S and G2, CtIP-mediated resection may occur in G1 via activation by polo-like kinase 3 (Plk-3) or during mitosis via Cdk1 (Peterson et al., 2011; Biehs et al., 2017). In G1, resection may reveal short sequence homologies adjacent to the DSB that may be repaired by microhomology-mediated end joining as discussed below. In mitosis, DSB resection blocks NHEJ while Cdk1 represses the activity of Rad51 thus postponing repair until the subsequent cell cycle.

While CtIP/Sae2 and MRN/X cleave in a 3’ to 5’ direction towards the DSB, long-range resection occurs in a 5’ to 3’ direction away from the break site. Long-range resection is carried out by the exonuclease Exo1 or the combined efforts of Dna2 (a partially redundant nuclease) and the Sgs1, Top 3, and Rmi1 (STR) complex at a rate of 4.4 kilobases per hour (Zhu et al., 2008). As ssDNA is exposed it is rapidly bound by Replication protein A (RPA). In addition to protecting ssDNA from degradation, RPA facilitates DNA unwinding by Sgs1 and directs the nuclease activity of Dna2 in a 5’ to 3’ orientation (Cejka et al., 2010). RPA-bound ssDNA also activates the PIKK ATR/Mec1. ATR/Mec1 phosphorylates CHK1 which in turns promotes the degradation of CDC25A (Costanzo et al., 2003; Flynn and Zou, 2011). Low levels of CDC25A delay cell cycle progression affording time for homology-directed repair (HDR).

Long-range resection proceeds over thousands of bases (Zhou et al., 2014) which raises the question: to what end? Limited resection is sufficient to expose sequence homology between meiotic chromosomes (Zakharyevich et al.,
Moreover, DSBs that use the sister chromatid as a template for repair successfully undergo HDR in the absence of Exo1 or Sgs1 (Westmoreland and Resnick, 2016). Longer ranges of resection might increase the specificity of the ssDNA/template interaction thereby facilitating the fidelity of the repair. However, ssDNA tracts are also prone to spontaneous base damage which might prevent the cell from repairing the lesion accurately (Chan et al., 2012). Another possible explanation is that extensive resection changes the physical properties of the chromatin fiber when RPA is exchanged for the recombination factor Rad51. As will be discussed below, Rad51 increases the stiffness of the DNA structure (van der Heijden et al., 2007) which could allow the break to more efficiently scan the genome for a non-sister template.

Rad51 filament nucleation occurs when a few Rad51 monomers bind to ssDNA and displace RPA. This process is mediated by BRCA2 (in mammals) and Rad52 (in yeast) which facilitate the loading of Rad51 onto ssDNA (Jensen et al., 2010; Sugiyama et al., 2002). As Rad51 polymerizes along the resected tract, the DNA is twisted such that the nucleotides are grouped into triplets – a conformation that facilitates Watson-Crick base pairing with a homologous template (Short et al., 2016). The Rad51 nucleoprotein filament subsequently searches for a homologous sequence. Given that end resection is favored in S and G2, the complementary strand may be readily found on the sister chromatid. Sister chromatids are held in close proximity by cohesin which loads onto DNA prior to replication and mediates connections between centromeres and sister chromatid arms (Tanaka et al., 1999). Moreover, cohesin accumulates at DSBs
in G2 to solidify sister chromatid tethering (Strom et al., 2004; Unal et al., 2004). Thus, HDR between sister chromatids is greatly preferred over recombination with the chromosomal homolog (Johnson and Jasin, 2000). However, DSBs generated in early S-phase may attempt to undergo HDR prior to replication completion. In this case, or when the sister chromatid is similarly damaged, HDR may require capture of the unbroken homologous chromosome.

Strand invasion of the Rad51 nucleoprotein filament and successful base-pairing with the complementary duplex DNA template creates a displacement loop (D-loop). The invading strand may be lengthened by DNA polymerases using the donor duplex as a template and subsequently reannealed to the second DSB end. Alternatively, the second DSB end may be captured into the donor duplex forming a four-way structure called a double Holliday junction (Hj). Double Hjs can be dissolved by the BLM/TOPOIII complex or cleaved by SLX1/SLX4 or GEN1, MUS81/EME1 (West et al., 2015). Upon resolution of these recombination intermediates, genome integrity is restored.

Although DNA end processing promotes repair fidelity by destining breaks for HDR, resection can also funnel DSBs into error prone pathways. DSBs arising in highly repetitive sequences can be repaired by single-strand annealing (SSA) of complementary strands (Bhargava et al., 2016). SSA causes a deletion rearrangement when stretches of uninterrupted homology anneal downstream of DSBs and the non-homologous 3’ ssDNA tails are cleaved. This process is carried out by Rad52 which facilitates the sequence homology search as well as strand annealing in cis (Rothenberg et al., 2008). Approximately 11% of the
human genome is comprised of *Alu* repeats which are found in cancer susceptibility genes including *MSH2*, *VHL*, and *BRCA1* (Zhang et al., 2011). Accordingly, SSA between breaks arising in these genes could contribute to cancer-associated intragenic rearrangements. Moreover, SSA between *Alu* elements on different chromosomes triggers chromosomal translocations (Elliott et al., 2005).

While SSA requires long-range resection to reveal complementary strands, minimal end processing may generate short sequence homologies adjacent to the break. Subsequent sequence annealing and ligation, termed microhomology-mediated end joining (MMEJ), restores a linear DNA molecule at the expense of genome integrity: regions of nonhomology flanking the original DSB are lost (Bennardo et al., 2008). The initial resection step is carried out by Mre11 and CtIP/Sae2 (Truong et al., 2013). However, unlike HDR, MMEJ is active throughout interphase. Unlike SSA, MMEJ may promote end joining of homologous sequences as short as a single nucleotide. Given its mutagenic potential, MMEJ must be tightly regulated and indeed RPA inhibits the annealing of small microhomologies in *S. cerevisiae* (Deng et al., 2014). Moreover, super-resolution microscopy indicates that the Ku complex can outcompete PARP1 for DSB ends in G1 cells which favors NHEJ over MMEJ (Yang et al., 2018). However, many cancers overexpress PARP1 which may shift repair from NHEJ to MMEJ and drive oncogenic rearrangements (Tobin et al., 2012).
Fig. 1-3 | Non-homologous end joining (NHEJ) and homology-directed repair (HDR) pathways compete for DNA double-strand breaks (DSBs).
Fig. 1-3 | Non-homologous end joining (NHEJ) and homology-directed repair (HDR) pathways compete for DNA double-strand breaks (DSBs).

a) NHEJ is active throughout interphase and rejoins simple breaks that require minimal end processing. DSB ends are rapidly bound by the Ku70/Ku80 heterodimer which facilitates the loading of DNA-PK and ARTEMIS. ARTEMIS trims short nucleotide overhangs to generate ends suitable for ligation by the combined efforts of XLF/XRCC4/LIG4. b) HDR is active throughout S and G2 phase when a sister chromatid is available to serve as a template for repair. HDR predominates when NHEJ fails or when DSBs have complex ends which cannot be ligated by NHEJ machinery. Complex ends are resolved when the Mre11-Rad50-Nbs1 complex binds upstream of the break end and nicks the 5’ dsDNA strand. Resection towards the DSB end is carried out by the combined efforts of CtIP and MRN. This generates a DSB end with a short single-stranded DNA overhang which is an avid substrate for the Exo1 nuclease leading to extensive end resection. Extensive end resection may alternatively be carried out by Dna2 (a partially redundant nuclease) and the Sgs1, Top 3, and Rmi1 (STR) complex (not shown). As single-stranded DNA is generated it is rapidly bound by RPA which is exchanged for Rad51. The Rad51 nucleoprotein filament subsequently searches for a homologous sequence typically found on the sister chromatid. Successful basepairing with a donor duplex generates a D-loop and double-holiday junction which may be unwound by the BLM/TOPOIII complex or digested by SLX1/SLX4 or GEN1, MUS81/EME1. (not pictured).
B. DNA double-strand breaks move

i. Fluorescently-tagged proteins reveal DSB dynamics

Following DSB generation cells form microscopically-detectable DNA repair foci. By fluorescently tagging repair and checkpoint proteins, the dynamics of repair foci may be observed by time-lapse microscopy. In budding yeast the movement of DSBs which repair primarily by HDR is commonly tracked by visualizing Rad52 which forms heptameric rings around ssDNA (Stasiak et al., 2000). In yeast Rad52 facilitates the displacement of RPA by Rad51 and mediates strand exchange prior to recombination (Mortensen et al., 1996; Bi et al., 2004). In mammalian cells Rad52 was originally thought to serve an auxiliary role to BRCA2. For example, Rad52 is required for Rad51 foci formation and gene conversion in BRCA2-deficient cells (Feng et al., 2011). More recently however, it has been shown that DSBs that fall in transcriptionally-active loci load Rad52 which facilitates the processing of proximate R-loops by XPG and the binding of BRCA1 (Yasuhara et al., 2018). This suggests a role for Rad52 in promoting HDR upstream of BRCA2 activity. Accordingly, fluorescently-tagged Rad52 and RPA have been used to track the motion of breaks undergoing HDR in mammalian cells (Karanam et al., 2012).

DSB dynamics in mammalian cells may also be monitored by fluorescently-tagged P53-binding protein 1 (53BP1). 53BP1 which lacks apparent enzymatic activity plays a complex role in regulating the choice between NHEJ and HDR. On one hand, 53BP1 shepherds essential factors for end-joining to the
DSB including Rif1, PTIP, and ARTEMIS which suppress recombination (Chapman et al., 2013; Wang et al., 2014). Moreover, 53BP1 is thought to act as a barrier to resection and require removal by BRCA1 for HDR (Bunting et al., 2010). On the other hand, 53BP1 was recently shown to localize to DSBs undergoing HDR (Ochs et al., 2016). Here, 53BP1 was proposed to regulate the extent of DSB end resection. The choice between 53BP1’s function in HDR and NHEJ may be regulated by its phosphorylation status. In S and G2, 53BP1 is dephosphorylated by the protein phosphatase PP4C which results in Rif1 release from the DSB and Exo1 recruitment (Isono et al., 2017). Taken together these data suggest that fluorescently-tagged 53BP1 may be used to track the movement of NHEJ breaks in G1 cells when HDR is inactive. However, 53BP1 cannot be used to distinguish NHEJ and HDR events in S and G2 when both pathways are operative.

   ii. **Assessing chromatin mobility: MSDs, D(t)s and α’s**

Chromatin motion is assessed using mean-square displacement (MSD) analysis which describes how repair foci move and the degree to which they explore their environment. MSD curves plot the average squared distance that foci travel over increasing time intervals. A freely diffusing particle is said to display Brownian motion and exhibits an MSD curve that increases proportionally with time (Zimmer and Fabre, 2018; Fig. 1-4a). In contrast, a particle moving in a directed manner will have an MSD curve with a positive curvature indicating extra displacements that exceed diffusive motion (Fig. 1-4b). Finally, particles moving
within a confined space will exhibit exponential MSD curves which reach a plateau (Fig. 1-4c).

MSD values are calculated using the equation $MSD = \langle (x(t+\Delta t)-x(t))^2 \rangle$, where $x$ is the position of the DSB focus and $t$ is time (Tarantino et al., 2014). One parameter that may be derived from the MSD curve is the diffusion coefficient $D(t)$. The $D(t)$ is approximated by the linear-weighted fit of the initial slope of the mean MSD curve and describes particle speed. A second parameter, the anomalous diffusion coefficient $\alpha$, can be obtained by plotting the log(MSD) against log(t). Similar to the shape of the MSD curve, $\alpha$ characterizes the degree to which a particle explores space. When $\alpha$ values are equal to 1 the particle is thought to move by normal diffusion. In contrast $\alpha$ values greater than 2 indicate directed movements while $\alpha$ values less than 1 indicate particle subdiffusion. Particle subdiffusion may be further classified as confined or anomalous. Confined subdiffusion reflects a structural impediment to motion akin to a spring dangling from a fixed object. Anomalous subdiffusion can reflect molecular crowding or other properties that restrain but not necessarily confine particle movement.
Fig. 1-4 | Types of particle movement.
Fig. 1-4 | Types of particle movement. a) Freely-diffusing particles display Brownian motion (top). The mean-square displacement (MSD) plots the average squared distance that particles travel over increasing time intervals. MSD curves that increases proportionally with time are characteristic of Brownian motion (bottom). b) Particle movements that exceed diffusion are called directed motions (top). MSD curves that increase without bounds as time approaches positive infinity are characteristic of particles with directed motion (bottom). c) Subdiffusive particles moving within a limited territory are said to display confined Brownian motion (top). Particles undergoing subdiffusion generate MSD curves that plateau at later time intervals.
iii. DSB movement and repair pathway choice

In diploid and haploid yeast, a single DSB generated by rare-cutting endonucleases triggers two responses in the genome: 1. Increased local mobility: the MSD of a tagged DSB is significantly higher than that of an undamaged region; 2. Increased global mobility: undamaged chromosomes also move albeit to a lesser degree (Dion et al., 2012; Miné-Hattab and Rothstein, 2012). The movements of DSB loci in yeast produce MSD curves that plateau at later time points indicating that damaged sites undergo confined Brownian motion (Dion et al., 2012). This increase in chromosome dynamics is time dependent and highly specific for DSBs. Immediately following DSB induction by HO cleavage DSB movements are actually more confined relative to undamaged chromosomes (Saad et al., 2014). Moreover, single-strand breaks (SSBs) induced by bleomycin or spontaneous lesions in S-phase cells do not exhibit increased motion (Dion et al., 2012; Dion et al., 2013). This suggests that the machinery that elicits chromatin movement is tightly regulated by the DNA damage response to occur downstream of DSB generation.

In mammalian cells DSB movements correlate highly with break complexity and the pathway of repair activated. In contrast to yeast, which repair I-SceI-induced DSBs by HDR, mammalian cells repair restriction endonuclease breaks almost exclusively by NHEJ (Manivasakam et al., 2001). Calculation of the MSD of I-SceI-induced DSBs in NIH3T3 cells indicates that these breaks exhibit similar dynamics as intact chromosomes (Roukos et al., 2013). Furthermore, DNA breaks induced by ultra-soft X-rays (USX), which yield
predominantly simple DSBs, are rapidly bound by the Ku70/80 complex and are repaired by NHEJ (Reynolds et al., 2012). Like I-Scel-induced breaks USX-induced DSBs remain positionally stable (Nelms et al., 1998). These studies and others have led to the puzzling contradiction that DSBs in yeast exhibit enhanced motion whereas DSBs in mammalian cells do not (Lemaître and Soutoglou, 2015). However, as will be discussed below, the dynamism of DSBs in mammalian cells is masked by the predominance of NHEJ and may be unmasked by specifically tracking breaks undergoing HDR.

DSB movements related to HDR may be visualized by the motion of damaged telomeres. Telomere ends are typically protected from DNA damage responses by the Shelterin complex which is comprised of six subunits including TRF1. Fusion of the nuclease Fok1 to TRF1 induces a DSB response at the telomere end (Tang et al., 2013). In the absence of telomerase, immortalized cells maintain telomeres via a process called alternative lengthening of telomeres (ALT) in which telomere ends recombine for homology-directed synthesis (Fasching et al., 2007). Similar to DSBs undergoing HDR in yeast, ALT telomeres are highly mobile in U2OS cells that express TRF1-FokI (Cho et al., 2014). Interestingly, broken telomeres that merge for recombination exhibit distinct motion properties: “incoming” telomeres have an $\alpha$ coefficient around 2 indicating directed movement while “recipient” telomeres have an $\alpha$ coefficient around 0.8 indicating confined motion. This suggests that telomeres undergoing homology search undergo rapid directional movements that culminate in synapsis.
DSB movements during HDR may also be visualized in mammalian cells by generating breaks with complex ends. While NHEJ prevails at clean DSBs, complex DSBs require end processing which generates a suitable substrate for HDR. For example, etoposide, a topoisomerase II poison, traps TopII adducts on DNA which may be excised by TDP-2 or resected by MRN and CtIP (Aparicio et al., 2016). In contrast DSBs induced by ionizing radiation (IR) are simpler and predominantly repaired by NHEJ in mammalian cells (Mahaney et al., 2009). Relative to the movement of IR-induced DSBs etoposide breaks exhibit substantially greater mobility in U2OS cells (Krawczyk et al., 2012). This further supports the notion that DSBs slated for HDR exhibit greater motion than those undergoing NHEJ. Finally, DSBs that fall within transcriptionally active genes load Rad51 in G2 and repair by HDR (Aymard et al., 2014). These HDR breaks migrate into clusters whereas NHEJ-prone DSBs do not (Aymard et al., 2017).

Often, DSB dynamics are visualized by single-particle tracking of chromosomes tagged by LacO and TetO arrays that do not inform on the mode of repair at the break site (Roukos et al., 2013). Taken together, these data highlight the need to consider repair pathway choice when interpreting the mobility of damaged chromosomes in mammalian cells. Furthermore, DSB motion may be tracked at different time scales which may reveal unique properties of chromatin movement. For example, when DSB movements are acquired at short time intervals (e.g. 10 ms) in yeast, broken chromosomes appear less mobile than undamaged loci (Miné-Hattab et al., 2017). When the same DSBs are imaged at longer time intervals (e.g. 1000 ms) the mobility of the
damaged sites is significantly greater. These apparently contradictory observations may be explained by the stiffening of the DNA structure upon Rad51 loading (Miné-Hattab et al., 2017). DNA stiffening decreases its motion which is detected on rapid time scales. In turn, DNA stiffening allows the DSB end to navigate the chromatin meshwork more effectively (akin to a needle in a ball of yarn) which is reflected by enhanced DSB motion on longer time scales.
Fig. 1-5 | Damaged chromatin moves.
**Fig. 1-5 | Damaged chromatin moves.**

| a) Following DSB end resection, ssDNA coated with Rad51 searches for an intact sequence to use for homology-directed repair. While a suitable sequence may be found on the homologous chromosome, the intact sister chromatid is the preferred template due to its proximity to the damaged site in mammalian cells. While HDR is restricted to S and G2 phase, condensed mitotic chromosomes are shown to clearly differentiate sister chromatids from chromosomal homologs. Red circles represent DSB sites while green circles represent intact homologous sequences. The path that DSB sites during homology search are shown (the red to green reflects DSB movement over time). Notably, in yeast, homology search often results in interhomolog pairing, which is reflected in the movement of the purple chromosomes. | b) Following DSB generation in yeast, mammalian cells, and Drosophila, DSBs cluster. DSB clustering occurs between damaged sites which coalesce to form a common focus. Here, DSB movements that result in clustering are shown by red tracks. Notably, DSB clustering does not reflect homology search as non-homologous chromosomes migrate into close proximity. | c) In Drosophila and mouse cells, heterochromatic (HC) DSBs migrate outside the HC compartment (mouse) or to the nuclear periphery (Drosophila). This movement is thought to prevent aberrant recombination between repetitive sequences within the HC domain. Here, HC is shown as dense clusters of DNA. Damaged sites are shown in red, and extrusion of damaged sites is indicated by paths that change from red to green over time. |
C. The genetic requirements for chromatin movement

i. DSB repair machinery

Many of the factors that carry out HDR are also required for chromosomal mobility. In budding yeast the mobility of damaged chromosomes requires several central HDR proteins including the Rad51 recombinase, the Rad54 ATPase, Rad52, and ATR (Dion et al., 2012; Miné-Hattab and Rothstein, 2012; Smith et al., 2018). Importantly, increased global mobility upon DSB generation is observed in cells expressing Rad51 mutants that lack recombinase activity yet form nucleoprotein filaments (Smith et al., 2018). This suggests that global chromatin motion is triggered downstream of resection yet upstream of recombination. Intriguingly, cells that lack both Rad51 and Rad52 exhibit elevated global mobility even in the absence of DNA damage. This effect is blocked by caffeine which inhibits Tel1/ATM and Mec1/ATR activity (Smith et al., 2018). These results indicate that 1.) the DNA damage checkpoint is sufficient to trigger chromatin movement and 2.) Rad52 might restrain chromatin movement until checkpoint activation promotes the loading of Rad51.

HDR machinery is also required for enhanced chromatin movement in other eukaryotic species. The diffusive and directed movements of ALT telomere DSBs require Rad51 in U2OS (Cho et al., 2014). Similarly, in Drosophila, DSB movement requires the activities of the end processing machinery, including MRN, CtIP, Exo1, and Blm (Chiolo et al., 2011; Caridi et al., 2018).
In contrast, NHEJ machinery may constrain movement. Upon DSB generation, DNA ends are initially tethered together by a complex containing Ku70/Ku80 and DNA-PK(cs) (Graham et al., 2016). Upon loss of Ku80, the motion of I-Scel-induced DSBs increases from 50 nm per minute to 80 nm per minute (Soutoglou et al, 2007). Moreover, cells depleted of DNA-PKcs exhibit increased movement and damaged chromosome pairing (Yamauchi et al., 2017). It is worth mentioning that knockdown of NHEJ machinery also increases the efficiency of HDR (Bennardo et al., 2008). Thus, the increase in movement observed upon depletion of Ku80 or DNA-PKcs might reflect a shift towards HDR-based motion at the break site.

ii. Nucleoskeletal anchors and cytoskeletal forces

In budding yeast chromosome ends are anchored in place by attachments to the SPB and nuclear periphery. The combined effect of disrupting these tethers is sufficient to induce chromatin mobility reminiscent of DSB motion (Strecker et al., 2016). Indeed, Cep3, a constituent of the kinetochore complex which mediates chromosomal attachment to the SPB, is phosphorylated in an ATR-dependent manner at S575. Non-phosphorylatable Cep3 S575A mutants exhibit no increase in the movement of HO endonuclease-induced DSBs. Moreover, Cep3 phosphorylation is required for the global increase in chromatin motion that follows DSB generation. Cep3 phosphorylation is thought to relax constraints on pericentromeric DNA thereby allowing broken chromosomes to explore more space (Lawrimore et al., 2017). Thus, DDR-mediated alterations in
chromosomal tethering to the SPB could promote increased local and global mobility.

Similarly, loss of telomere constraints at the nuclear envelope might enhance DSB motion. Telomeres attach to the nuclear periphery via the Ku70/80 complex as well as Sir4 and Esc1 (Taddei et al., 2006, Fig 1-2). DSB generation in budding yeast has been shown to release telomeres from the nuclear periphery which diffuse into the interior (Lawrimore et al., 2017). This migration is mediated in part by microtubule polymerization which is thought to transmit forces generated in the cytoplasm to chromatin via the nucleoskeleton. In fact, microtubule polymerization is required for the mobility of uncapped telomeres which resemble DSB ends in mammalian cells (Lottersberger et al., 2015). In this setting, telomere movements also require components of the linker of the nucleoskeleton, and cytoskeleton (LINC) complex including SUN1/2 and nesprin-4 as well as the plus-ended microtubule motor kinesin-1. These data suggest that cytoplasmic microtubules increase DSB dynamics at LINC sites.

As will be discussed below, cytoskeletal forces may also be generated within the nucleus. Indeed, small microtubules have been shown to polymerize in the nucleoplasm in response to DNA damaging agents (Oshidari et al., 2018). Movement of damaged chromosomes is facilitated by the Kar3 kinesin motor which captures broken segments of DNA and transmits them along microtubules to the nuclear periphery for repair. These movements specifically affect DSBs destined for break-induced replication which resolves one-ended DSBs by
homologous recombination. Indeed, previous studies have found that BIR breaks relocalize to the nuclear pore sub-complex NUP84 for repair (Chung et al., 2015).

iii. Chromatin remodelers

Chromatin structure controls the accessibility of the nucleotide sequence to essential DNA transactions including replication, transcription and repair. For example, nucleosomes may block accessibility of resection machinery and other repair factors to DSB ends (Adkins et al., 2013). Cells overcome this barrier by recruiting a variety of chromatin remodeling complexes including the INO80, RSC, SWI/SNF and SWR1-C complexes which reorganize nucleosomes in an ATP-dependent manner (Hauer and Gasser, 2017). Chromatin decompaction following DSB generation increases the movement of chromatin both locally at the break site and globally in S. cerevisiae (Neumann et al., 2012; Seeber et al., 2013a). This movement requires the ATPase activity of Arp8, an actin-related protein bearing 30% homology to β-actin (Muller et al., 2005). In mouse cells however, chromatin decompaction at DSB sites is not linked to increases in chromosome mobility (Kruhlak et al., 2006). In this setting, limited DSB motion may reflect the propensity of breaks to undergo NHEJ in mammalian cells.
Fig. 1-6 | Genetic Requirements for DSB movement.
**Fig. 1-6 | Genetic Requirements for DSB movement.** A mammalian cell nucleus is shown with damaged and undamaged sites indicated by red and yellow circles, respectively. Dashed lines indicate sister chromatids while homologous chromosomes share identical colors. First, homology search in yeast and mammalian cells requires the Rad51 recombinase (box, top left). DSB movements in yeast also require ATR and the Rad54 ATPase (not shown). In Drosophila, the activities of MRN, CtIP, Exo1, and Blm are required for DSB relocation outside the heterochromatic domain (not shown). Second, microtubule (MT) polymerization in the cytoplasm transduces forces to damaged chromatin that generates movement (box, top right). MT-driven forces are relayed via the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex. Third, DSBs recruit chromatin remodeling complexes which reorganize nucleosomes thereby increasing access of repair machinery to the damaged site (box, bottom left). Chromatin decompaction facilitated by chromatin remodelers promotes chromosome mobility. Finally, DSBs that undergo NHEJ have limited mobility (box, bottom right).
D. Types of DSB movement

i. Homology search
At baseline, yeast chromosomes have fewer constraints to motion and are accordingly more dynamic than mammalian chromosomes (Marshall et al., 1997; Chubb et al., 2002). Yeast nuclei are also approximately 80 times smaller than human nuclei (Miné-Hattab and Rothstein, 2013). Thus, increased DSB movement during HDR allows the break to efficiently scan the entire genome for a non-sister template (Fig. 1-5a). Indeed, in the absence of a DSB, homologous chromosomes are paired in less than 3% of cells (Miné-Hattab and Rothstein, 2012). However, within two hours of DSB generation, homologous loci co-localize in 30% of cells which coincides with the appearance of repair products. In mammalian cells ALT-driven movements offer the clearest evidence of interhomolog pairing between telomere ends (Cho et al., 2014). As will be discussed below, chromosomally-induced DSBs have more limited mobility which promotes clustering between breaks that are in close proximity.

ii. DSB clustering
Clustering between non-homologous chromosomes is a general organizing principle in yeast and mammalian cells that brings together genomic loci that share a common function. Irreparable DSBs in yeast coalesce to occupy a single locus (Lisby et al., 2003). Similarly, damaged chromatin domains induced by alpha particles in HeLa cells merge into clusters (Aten et al., 2004). Moreover,
DSBs induced by the AsiSI restriction enzyme cluster as well (Caron et al., 2015). DSB clustering requires the activity of ATM as well as the MRN complex and has been proposed to preferentially coalesce DSBs slated for HDR (Aymard et al., 2017). However, the role DSB clustering plays in repair is poorly defined. DSB clustering may concentrate the repair machinery in centers to regulate repair (Fig. 1-5b). This concept draws from other examples of clustering in biology. For one, clustering of Ras molecules within the plasma membrane concentrates MAPK cascade proteins enabling signaling proportional to EGF input (Tian et al., 2007). Alternatively, clustering may sequester DSBs undergoing HDR from those undergoing NHEJ and MMEJ to prevent mutagenic repair events (Marnef and Legube, 2017). On the other hand, it is possible that DSB clustering is a random event facilitated by electrostatic interactions between PARylated DSBs (Altmeyer et al., 2015). Finally, DSB clustering may positively contribute to the formation of chromosomal translocations as will be discussed below.

iii. **DSB relocalization**

In addition to facilitating homology search and clustering, chromosomal movements may transit DSBs to sites permissive for repair. This is particularly true for DSBs arising in repetitive sequences which migrate far from their origin to avoid aberrant recombination (Fig. 1-5c). In *S. cerevisiae* ribosomal DSBs relocalize to an extranucleolar site in a process that requires Mre11 and Smc5-Smc6 (Torres-Rosell et al., 2007). Failure to reposition ribosomal DSBs leads to
loss of rDNA repeat sequences. Similarly, DSBs induced in mouse pericentromeres relocate to the periphery of heterochromatin compartment (Tsouroula et al., 2016). Notably, these breaks remain positionally stable in G1 cells and repair by NHEJ whereas breaks undergoing HDR move in G2. Finally, in *D. melanogaster* DSBs arising within heterochromatin migrate to the nuclear periphery in a process requiring the activities of CtIP, Exo1, and more recently the Arp2/3 complex, as will be discussed below (Chiolo et al., 2011; Caridi et al., 2018).

In addition, DSB localization to the periphery may position breaks near nuclear pores which act as hubs for repair. In *S. cerevisiae* DSBs that lack a homologous donor transit to the nuclear envelope where they associate with the Nup84 nuclear pore complex and the small ubiquitin-like modifier (SUMO)-dependent ubiquitin ligase Slx5/Slx8 (Nagai et al., 2008). This relocalization requires the activities of Mec1/ATR and Tel1/ATM. Ubiquinylation at the nuclear pore is proposed to target sumoylated proteins on irreparable DSBs for degradation thereby facilitating repair. Indeed, polySUMOylation of persistent DSBs leads to Slx5/Slx8 recruitment which transits breaks to the periphery (Horigome et al., 2016). There DSBs may become tethered via associations with the SUN protein Mps3p which spans the inner nuclear membrane (Oza et al., 2009). Ultimately, DSB relocalization to the periphery could serve as a requisite step in the resolution of slowly-repaired breaks by HDR as evidenced by the requirement of DSBs to associate with the LINC complex prior to recombination in *S. pombe* (Swartz et al., 2014). In mammalian cells, nuclear pores also
represent permissive sites for HDR (Lemaître et al., 2014). In contrast to DSBs in *Drosophila*, however, heterochromatic DSBs generated in the nuclear lamina fail to migrate to nuclear pores in human cells. Rather, HDR repression at LAD-associated DSBs may limit recombination between repetitive sequences thereby avoiding genome instability.

### E. Translocations: an undesired consequence of DSB motion?

Translocations are deleterious genomic rearrangements that form by the joining of chromosome segments. Frequently used to stratify tumors in terms of prognosis and risk of transformation, translocations drive oncogenesis via the formation of chimeric genes or deregulation of tumor suppressors. For two DSBs of distinct chromosomes to juxtapose they must first be brought into close proximity. Single particle tracking of translocating DSBs in NIH3T3duo cells reveals significantly faster chromosomal movements relative to non-translocating DSBs that presumably undergo NHEJ (Roukos et al., 2013). Most translocations occur between proximate DSBs although clustering between distant chromosomes is also observed.

Intriguingly, DSBs arise proximate to transcription start sites in highly transcribed genes, which are frequent substrates for oncogenic translocations (Klein et al., 2011; Schwer et al., 2016). For example, IgH, IgK, and IgL loci are known to translocate with c-myc in the development of Burkitt’s lymphoma (Schmitz et al., 2014). Transcribed genes are brought into close proximity via
their association within a limited number of specialized transcription factories (Schoenfelder et al., 2010). The basis for the clustering of actively transcribed genes is unknown; however, the formation of transcription factories and repair foci both require intrinsically disordered proteins (IDPs) which aggregate and phase separate into liquid droplets (Altmeyer et al., 2015; Chong et al., 2018; Sabari et al., 2018). It is tempting to speculate that some of the factors that drive DSB motion are shared with transcriptional machinery. Indeed, DSBs in transcriptionally-active genes cluster (Aymard et al., 2017). However, whether these breaks cluster (or translocate) because of their transcriptional status or repair pathway choice remains to be elucidated.

3. A ROLE FOR POLYMERIZED ACTIN IN DNA TRANSACTIONS

Globular actin is a core component of the cytoskeleton that polymerizes into microfilaments which provide structure to eukaryotic cells and generate forces essential for movement. Microfilaments assemble into long interwoven chains known as a filamentous actin (F-actin). The critical concentration for actin polymerization is higher at the pointed (minus) end of the microfilament (~0.7 μM) than at the barbed (plus) end (~0.1 μM) (Lodish et al., 2000). Accordingly, microfilaments polymerize actin monomers up to ten times faster at the barbed end relative to the pointed end. Following filament incorporation actin subunits hydrolyze ATP (Blanchoin and Pollard, 2002). Actin filaments coordinate depolymerization by incorporating recently dissociated subunits from the pointed
end into the barbed end. This “treadmilling” behavior allows cells to push structures like lamellipodia which protrude against the plasma membrane during cell migration (Koestler et al., 2009).

De novo actin filaments assemble when actin dimers and trimers form a polymerization seed. The formation of seeds, however, is highly unstable which prevents spontaneous polymerization in the cell. Furthermore, most actin monomers are sequestered from polymerization by binding to thymosin β4. This maintains a pool of intracellular unpolymerized actin. Cells overcome energetic barriers to polymerization by employing distinct nucleation factors including the Arp2/3 complex, the formin family of proteins, Spire 1 and 2, Cobl, Lmod, TARP, and VopL/VopF. Actin filament nucleators exert significant influence over the structure of the actin arrays they generate. For the sake of brevity, only the Arp2/3 complex and the formin family of proteins will be discussed here. Emphasis is placed on the regulation of Arp2/3 as its activity is central to the focus of this thesis.

A. Actin nucleators and nucleating promoting factors

i. WASP and ARP2/3

The Arp2/3 complex contains seven proteins including the actin-related proteins Arp2 (44-kD) and Arp3 (47-kD) which share 50% homology with conventional actin (42-kD). In human cells, Arp2 and Arp3, together with the Arp complex subunits ARPC1, ARPC2, ARPC3, ARPC4, and ARPC5, mediate the formation
of branched actin structures. Arp2/3 binds to the side of a pre-existing mother filament and polymerizes daughter filaments at 70-degree angles. In addition to its nucleation activity, Arp2/3 crosslinks newly nucleated actin filaments with older ones generating a dendritic network of Y-branched structures (Mullins et al., 1998). Polymerization at the barbed ends of this network generates propulsive forces that drive movement. For example, Arp2/3 facilitates cell migration by extending lamellipodia at the leading edge (Ridley, 2011).

In its inactive state, Arp2 and Arp3 lie end-to-end in a splayed conformation (Robinson et al., 2001). Upon activation by nucleation promoting factors (NPFs), Arp2 turns 25 angstroms into position next to Arp3 (Rouiller et al., 2008). This short-pitch conformation forms an Arp2-Arp3 dimer that resembles two consecutive actin subunits (Fig. 1-6a). Like a polymerization seed, Arp2-Arp3 dimers accelerate the formation of nascent filaments that are stabilized at their pointed end by the mother filament.

The other five subunits within the Arp2/3 complex serve distinct structural roles. Arp2/3 binds the mother filament via ARPC2 and ARPC4 which form the backbone of the complex. Mutations in ARPC2 and ARPC4 that reduce their affinity for the mother filament reduce the nucleating activity of the complex (Goley et al., 2010). Thus, Arp2/3 must associate with the side of a pre-existing filament to trigger polymerization. In contrast, ARPC3 stabilizes the inactive conformation of Arp3 while ARPC5 wraps around Arp2 thereby tethering Arp2 to the rest of the complex (Rouiller et al., 2008). Finally, ARPC1 makes minimal
contact with the mother filament and instead regulates Arp2/3 activity by binding NPFs as will be discussed below.

While Arp2/3 is classically composed of seven subunits, variations in subunit isoform and phosphorylation status impact the complex’s ability polymerize actin. Complexes containing ARPC1B and ARPC5L polymerize actin far more efficiently than complexes composed of their paralogs of ARPC1A and ARPC5 (Abella et al., 2016). On the other hand, Arp2/3 complexes that lack ARPC1 and ARPC5 subunits have decreased efficiency of actin nucleation yet retain the ability to cross-link actin filaments (Gournier et al., 2001). Finally, the phosphorylation of several residues within the complex are thought to modify the interactions between subunits. For example, salt bridges between sites T237 and T238 on Arp2 and R105 and R106 on ARPC4 stabilize the Arp2/3 complex in an inactive conformation (Narayanan et al., 2011). Phosphorylation of the Arp2 sites by the Nck-interacting kinase NIK may destabilize these interactions thereby facilitating Arp2/3 activation by NPFs (LeClaire et al., 2008; LeClaire et al., 2015).

Unaided, the Arp2/3 complex is a poor actin nucleator and necessitates activation by a class of NPFs known as the Wiskott-Aldrich syndrome protein (WASP) family which include WASP, N-WASP, SCAR/WAVE, WASH, WHAMM, and JMY. Members of the WASP family share divergent N-terminal domains yet significant C-terminal homology. In particular, the VCA (V: Verprolin homology, C: Connector, A: Acidic) motif on the C-terminus, which activates Arp2/3, is highly conserved. Arp2/3 nucleation requires binding of two VCA domains (Padrick et al., 2011). Each VCA stimulates Arp2/3 activity several ways. First,
the VCA domain increases the association rate of Arp2/3 with the sides of mother filaments ensuring the production of branched filaments (Smith et al., 2013a). Second, Arp3 and ARPC1 bind to the C and A motifs on WASP, respectively (Luan et al., 2018). This interaction is thought to displace the C-terminal tail of Arp3 from its barbed end groove which allows Arp2 and Arp3 to move into a short pitch conformation (Rodnick-Smith et al., 2016a). Third, WASP delivers monomeric actin to Arp2/3 via the V domain to facilitate daughter filament growth (Padrick et al., 2011). However, the delivery of actin monomers may not be essential for nucleation as chemically crosslinking Arp2 and Arp3 bypasses the requirement for WASP (Rodnick-Smith et al., 2016b). Finally, WASP detaches from the Arp2/3 complex to enable filament growth (Smith et al., 2013b). This detachment may be mediated by cortactin, an NPF, which potently activates Arp2/3 after dislodging WASP (Helgeson and Nolan, 2013).

WASP typically locks its VCA motif in an inactive state by binding its N-terminal GTPase-binding domain (GBD) to the C-terminal domain. The WASP-interacting protein (WIP) stabilizes this autoinhibitory conformation and localizes inactive WASP to potential sites of Arp2/3 nucleation (Martinez-Quiles et al., 2001; Ho et al., 2004; Chou et al., 2006). There, WASP is disinhibited upon the cooperative binding of phosphatidylinositol (4,5)-bisphosphate (PIP2) to the BR domain and activated Cdc42 to the GBD domain (Higgs and Pollard, 2000). Subsequent Y291 phosphorylation in the GBD domain by non-receptor kinases Btk, Fyn or Hck liberates the VCA domain for Arp2/3 activation (Cory et al., 2002; Blundell et al., 2009). Alternatively, WASP may be released from autoinhibition
by Nck which cooperatively binds WASP with PIP2 to activate Arp2/3 independently of Cdc42 activity (Rohatgi et al., 2001). Finally, WASP degradation is carried out by the E3 ligase Cbl-b which targets WASP for ubiquitylation by c-Cbl (Reicher et al., 2012).

WASP and Arp2/3 may be inactivated by well-characterized small molecule inhibitors. The Arp2/3 inhibitor CK-666 rests in the binding pocket between Arp2 and Arp3 thereby stabilizing the Arp2/3 complex in an open, inactive conformation (Nolen et al., 2009; Hetrick et al., 2013). CK-548, a different Arp2/3 inhibitor, inserts into the hydrophobic core of the Arp3 subunit and prevents its dimerization with Arp2. CK-689 is an inactive structural analog of CK-666. Wiskostatin is a small molecule inhibitor that locks WASP in its autoinhibited conformation thus abrogating activation of the Arp2/3 complex (Peterson et al., 2004). Since wiskostatin also interacts with and inhibits the WASP cytoplasmic homolog N-WASP, WASP may be directly targeted by RNA interference.
Fig. 1-7 | Regulation of actin polymerization by WASP, Arp2/3, and related proteins.
Regulation of actin polymerization by WASP, Arp2/3, and related proteins. The Arp2/3 complex nucleates a branched network of actin filaments at 70-degree angles. These filaments generate forces classically at the leading edge. Arp2/3 filament assembly is regulated by the Wiskott Aldrich Syndrome protein family including WASP (pictured) and N-WASP, SCAR/WAVE, WASH, WHAMM, and JMY (not shown). WASP is directed to sites of Arp2/3 nucleation by WASP-interacting protein (WIP) which stabilizes WASP in an inactive conformation. Release of WASP from WIP promotes WASP-Arp2/3 interactions. WASP triggers a conformational change in Arp2/3 structure allowing it to adopt a short pitch conformation between the Arp2 and Arp3 subunits. WASP subsequently delivers actin monomers to Arp2/3 forming the pointed end of a nascent filament. Older actin subunits are bound by ADP and are preferentially targeted for depolymerization by ADF/Cofilin. Cofilin increases the separation between ADP-actin monomers within the filament thereby promoting disassembly. Alternatively, the protein GMF may sever actin filaments at the base of the Arp2/3 complex. Finally, excess elongation of actin filaments is prevented by capping protein which binds the barbed end of the filament. Collectively, the actions of capping protein, cofilin, and GMF maintain a steady pool of actin monomers that may be accessed at sites of active polymerization.
ii. Regulation of WASP-Arp2/3 dynamics: Cofilin and Capping protein

Following, Arp2/3 activation, ATP hydrolysis on the Arp2 subunit is thought to facilitate the dissociation of the complex from the mother filament (Nolen et al., 2004; Dayel and Mullins, 2004; Ingerman et al., 2013). While Arp2/3 branches dissociate with a half-life of 28 minutes in vitro (Goley et al., 2010), the disassembly of Arp2/3-polymerized structures is greatly potentiated by the severing of branched actin by actin depolymerizing factor (ADF)/cofilin which disassembles bare filaments at a rate of 7 subunits per second (Wioland et al., 2017). Cofilin weakens actin filaments by opening the nucleotide-binding cleft between actin monomers thereby disrupting interactions between subunits (Fan et al., 2013). Cofilin specifically severs filaments containing ADP-actin due to their propensity to undergo twisting fluctuations that allows cofilin to dislodge subunits (Tanaka et al., 2018). Thus, cofilin plays a critical role in accelerating actin filament turnover and dynamically reorganizing the dendritic network.

Arp2/3 disassembly is also potentiated by glia maturation factor (GMF) which dislodges ADP-Arp2 from the daughter filament (Boczkowska et al., 2013).

While cofilin promotes actin disassembly at pointed ends, filament growth is regulated at the barbed end by capping protein. Capping protein is a heterodimer composed of CapZα and CapZβ that prevents the loss or addition of actin subunits at the barbed end. By limiting filament growth, capping protein increases the efficiency of Arp2/3-driven movements by funneling free monomers to sites of active Arp2/3 assembly (Cooper and Sept, 2008).
iii. The Formin family

In contrast to the Arp2/3 complex, the formin family nucleates short actin filaments that assemble into actin cables or contractile rings. Formins are defined by their catalytic formin homology 2 (FH2) domains and are divided into two families: Formin (FMN) and Diaphanous (Dia). Dia1 (mDia1 in mice) is typically locked in an autoinhibited state via interactions between its N- and C-termini (Goode and Eck, 2007). mDia1 is activated by GTP-bound Rho GTPase. Upon dimerization, formins nucleate filaments by stabilizing actin dimers and trimers. As filaments grow, formins travel along with the barbed end in a process termed leaky capping. Formin-mediated actin polymerization drives the formation of filopodia (thin membrane protrusions), as well as stress fibers that mediate cell adhesion, and the cytokinetic actin ring that cleaves dividing cells.

B. Actin monomers and polymers drive nuclear processes

Since actin was isolated from nuclear extracts of the slime mold Physarum polycephalum, it has been identified in nuclei throughout eukarya (Lestourgeon et al., 1975). Given its abundance in the cytoplasm, biochemical studies implicating actin in nuclear transactions were initially panned as artifacts of contamination (Egly et al., 1984). This skepticism gradually abated as the discovery that actin associates with chromatin remodeling complexes and RNA polymerases renewed interest in the field.
Actin is shuttled into and out of the nucleus by importin-9 and exportin-6, which bind profilin-actin and cofillin-actin complexes, respectively (Stüven et al., 2003; Bohnsack et al., 2006). While nuclei without exportin-6, like *Xenopus laevis* oocytes, contain a meshwork of polymerized actin, most cells have low levels of filamentous actin indicating a pool of unpolymerized monomers. However, the machinery that assembles actin in the cytoplasm is also found in the nucleus (Weston et al., 2012; Virtanen and Vartiainen, 2017). Specifically, the Arp2/3 complex, WASP, and formins are located in both cellular compartments (Wu et al., 2006; Yoo et al., 2007; Taylor et al., 2010; Belin et al., 2015).

How cells use actin polymerization machinery to facilitate DNA transactions is beginning to emerge. Molecular tools for detecting intranuclear actin dynamics have been advanced by the development of fluorescent probes which bind actin monomers (Melak et al., 2017). While phalloidin which binds F-actin remains the gold standard for visualizing endogenous actin structures, it does not permeabilize cell membranes making the detection of intranuclear structures difficult. Moreover, phalloidin stabilizes native F-actin structures which limits its application to fixed samples. Similarly, overexpression of actin-detecting probes like Utrophin, F-tractin, and LifeAct have been criticized for altering actin dynamics in mammalian cells and promoting the formation of filaments (Du et al., 2015). In contrast, actin-directed chromobodies have been successfully employed to study nuclear actin without apparent effects on actin polymerization (Plessner et al., 2015). Nuclear actin dynamics may be specifically visualized by tagging the fluorescent chromobody with a nuclear localization sequence (NLS).
Alternatively, NLS-actin chromobodies may also express a nuclear export sequence (NES) (Baarlink et al., 2017). This facilitates the simultaneous visualization of cytoplasmic actin and prevents intranuclear accumulation of the probe. Notably, no studies to date have reported changes in native actin dynamics in cells expressing the chromobody construct.

i. DNA repair

Nuclear actin structures arise in mammalian cells following DNA damage with a variety of genotoxic agents including neocarzinostatin (NCS), a radiomimetic antibiotic and methyl-methane sulfonate (MMS), an alkylating agent (Belin et al., 2015). Different classes of nuclear actin structures are observed including long filaments and amorphous clusters. Knockdown of formin-2 (FMN2) decreases the fraction of damaged cells with long filaments but does not affect the incidence of cells with amorphous clusters. Notably, filaments did not colocalize substantially with 53BP1 foci which mark DSBs undergoing NHEJ in G1 and G2 cells or HDR in G2 cells. In this setting, the colocalization of actin clusters with repair foci has not been tested, nor has the effect of Arp2/3 inhibition on filament formation been shown.

In *D. melanogaster* DSBs arising within heterochromatin migrate to the nuclear periphery in a process requiring the activities of CtIP, Exo1, and MRN (Chiolo et al., 2011). This transit occurs prior to Rad51 loading and is thought to prevent aberrant recombination between repetitive sequences inside the heterochromatic compartment (HC). Consistent with studies in mammalian cells,
*D. melanogaster* cells expressing the actin chromobody construct polymerize nuclear actin filaments upon DSB generation (Caridi et al., 2018). These filaments are nucleated by the Arp2/3 complex which colocalizes with DSB sites within the HC. Notably, treatment with CK-666 or Arp2/3 RNA interference blocks the movement of heterochromatic DSBs and reduces the clustering of euchromatic DSBs induced by IR. These data suggest that nuclear Arp2/3 polymerizes actin to move DSBs to more permissive sites for HDR.

Importantly, the relocalization of heterochromatic DSBs to the periphery is an example of directed movement while the clustering of euchromatic DSBs occurs by confined Brownian motion. While Arp2/3 activity is solely required to promote clustering, the directed movement of heterochromatic breaks also requires the activities of myosin I and V which colocalize with HC sites. Myosin I is thought to serve as molecular dock while myosin V directs processive movement along actin filaments (Mehta et al., 1999; McIntosh and Ostap, 2016). Accordingly, the directed motion of heterochromatic breaks may simply reflect their association with myosins that transit along Arp2/3-polymerized filaments.

**ii. Chromatin remodeling**

Cells increase chromatin accessibility by recruiting a variety of chromatin remodelers including the INO80, RSC, SWI/SNF and SWR1-C complexes which reorganize nucleosomes in an ATP-dependent manner (Hauer and Gasser, 2017). All of the aforementioned complexes are comprised of distinct
combinations of actin related proteins (ARPs). For example, INO80 contains the actin subunit, Arp4, Arp5, and Arp8, while RSC contains Arp7 and Arp9 (Dion et al., 2010). ARPs are required for the proper architecture and function of their corresponding complex. For example, nucleosome eviction by INO80 requires the ATPase activity of Arp8. Notably, Arp2 and Arp3 which comprise the Arp2/3 complex are not subunits of chromatin remodelers. Moreover, while the INO80 complex contains the actin subunit, the barbed end of the monomer is embedded within the complex. This suggests that actin polymerization is not required for INO80 activities. Indeed, application of Latrunculin A, which inhibits actin polymerization, blocks INO80 complex activity by sequestering actin monomers during remodeling complex assembly (Spichal et al., 2016).

iii. Chromatin organization and decompaction

In early G1, the genome undergoes significant reorganization to reestablish the chromosomal topology that characterizes an interphase nucleus. Concordantly, cells undergo nuclear expansion and chromatin decompaction. Following mitotic exit, NIH3T3 cells expressing the actin chromobody construct polymerize transient nuclear actin filaments (Baarlink et al., 2017). These filaments abut the nuclear membrane forming small protrusions that increase nuclear volume. Moreover, overexpression of exportin 6, which depletes intranuclear levels of monomeric actin, or R62D actin, a dominant-negative mutant, delays the decompaction of chromatin in early G1. Interestingly, the nucleator that drives actin polymerization upon mitotic exit remains to be discovered. However,
nuclear volume expansion via actin polymerization requires the activity of the actin depolymerizing factor cofelin. Cofilin by preferentially severing older filaments may funnel nuclear actin monomers to sites of active polymerization thereby promoting nuclear growth.

iv. Transcription

Actin interacts with RNA polymerases I, II, and III, promotes transcription by all three polymerases, and drives the movement of transcribed genes (Percipalle and Visa, 2006). However, the link between actin-driven chromosomal movements and transcription efficiency remains poorly understood. Tethering of the VP16 transcriptional activator to an engineered chromosomal site in C6 cells induces the locus’ migration from the nuclear periphery to the interior (Tumbar and Belmont, 2001). This movement can be abrogated by the expression of a dominant-negative mutant actin (G13R) tagged to NLS which prevents nuclear actin polymerization by binding wild-type monomers (Chuang et al., 2006). Similarly in yeast, targeting VP16 to chromosomal loci increases chromosomal mobility (Neumann et al., 2012). Movement of transcribed loci by actin may facilitate their clustering within specialized transcription factories (Schoenfelder et al., 2010). In support of this idea, the Arp2/3 complex, WASP, and F-actin localize upstream of promoters of actively transcribed genes (Taylor et al., 2010). Moreover, expression of dominant-negative mutant N-WASP or RNA interference against Arp2/3 inhibits RNA polymerase-II-dependent transcription in HeLa cells (Wu et al., 2006; Yu et al., 2007). Given that DSBs in transcriptionally-active
genes cluster and are frequent substrates for chromosomal translocation (Gothe et al., 2018), the interplay between damaged DNA dynamics and transcription-related clustering is ripe for investigation.

Nuclear actin polymerization may also indirectly increase select transcriptional processes by releasing transcription factors from the inhibitory effects of monomeric actin. In response to serum stimulation, quiescent fibroblasts expressing LifeAct polymerize actin filaments via the formins mDia1 and mDia2 (Baarlink et al., 2013). Actin polymerization releases the megakaryocyte acute leukemia (MAL) protein from actin monomers which in turn activates serum response factor (SRF) to drive transcription. SRF is also induced by cell spreading which activates mDia formins to polymerize nuclear actin (Plessner et al., 2015).
Fig. 1-8 | Functions for nuclear actin.
Fig. 1-8 | Functions for nuclear actin. A mammalian cell nucleus is shown with circles denoting genomic loci undergoing the genomic transaction listed above its respective box. Arrows indicate movement of genomic loci. First, transcriptionally active genes cluster in the nucleus forming transcription factories (box, upper left). WASP, Arp2/3, and filamentous actin localize at promoter regions and loss of nuclear actin polymerization impedes movement of sites undergoing transcription. Second, formins polymerize actin following serum stimulation (shown as red circle in box, lower left). At rest, actin monomers bind and suppress megakaryocyte acute leukemia MAL. Upon nuclear actin polymerization MAL is released and activates serum response factor (SRF) to drive transcription (not shown). Third, heterochromatic DSBs recruit the Arp2/3 complex and myosin I and V which move damaged DNA to the nuclear periphery (box, upper right) in Drosophila. Arp2/3 (but not myosin) is also required for the clustering of euchromatic DSBs (not shown). Fourth, DNA damage induces the formation of nuclear actin filaments in mammalian cells (box, lower right). Nuclear actin filaments have elongated or globular morphology. Elongated filaments are polymerized by formins while globular filament structures are nucleated by unknown factors.
4. WASP AND ARP2/3 IN HEALTH AND DISEASE

Actin dynamics facilitate essential cellular transactions including motility, signaling, vesicular transport, attachment/adhesion, and infrastructure. Many of these processes are tightly regulated by WASP and Arp2/3 which organize actin polymerization at precise spatial and temporal coordinates. WASP has several homologs including N-WASP, which is expressed ubiquitously throughout tissues. In contrast, WASP is classically described as functioning exclusively in hematopoietic cells (Millard et al., 2004). However, recent evidence suggests that WASP is expressed, albeit at lower levels, within the nuclei of a variety of cell types including neurons, fibroblasts, and myocytes (Uhlen et al., 2015). While few studies to date have explored WASP’s activities in these cells, the clinical manifestations of mutations in WASP reflect its cytoplasmic and nuclear functions in myeloid and lymphoid lineages.

A. Wiskott-Aldrich Syndrome

The WASP gene is located on the short arm of the X chromosome at position Xp11.22-p11.23. More than 300 distinct mutations that produce defects in WASP expression have been identified, and most localize to exons 1-4 (Massaad et al., 2013). Mutations that completely abrogate WASP expression cause the X-linked recessive Wiskott-Aldrich Syndrome (WAS), whereas variant alleles that moderately decrease WASP induce X-linked thrombocytopenia (XLT). WAS male
children exhibit microthrombocytopenia, T cell lymphopenia, autoimmunity, and are particularly predisposed of lymphoma and leukemia. Female carriers of WASP mutations generally exhibit few, if any, hematological symptoms due to selective inactivation of the defective X chromosome in hematopoietic cell lines (Greer et al., 1989). This may reflect positive selection of cells expressing the normal X-chromosome during early stages of hematopoietic differentiation (Lacout et al., 2003). However, preferential inactivation of the healthy X chromosome has been observed in some females which induces WAS symptoms (Andreu et al., 2003). In contrast, patients with XLT exhibit milder thrombocytopenia, immunodeficiency, and autoimmunity.

The immunodeficiency observed in WAS patients predominantly arises from abnormal development of T cells, although defects in B-cells, NK cells, dendritic cells, and macrophages are also observed (Massaad et al., 2013). CD4+ T-cells require WASP-activated Arp2/3 to polymerize actin at the immunological synapse upon stimulation (Calvez et al., 2011). In this setting, actin polymerization serves to cluster lipid rafts bearing signaling molecules at the cell surface where they mediate T-cell receptor functions (Dupre et al., 2002). Moreover, nuclear WASP, Arp2/3 and F-actin localize to the TBX21 promoter in T helper 1 (T_H1) cells where they might facilitate gene transcription required for normal T_H1 activity (Taylor et al., 2010). Accordingly, the immunodeficiency and autoimmunity in WAS largely reflects impaired T cell activation and decreased T_H1 cell function, respectively. Finally, WAS-derived T cells, B cells, NK cells, and macrophages exhibit few surface microvilli and decreased migration thus
reflecting WASP’s role in reorganizing the actin cytoskeleton at the leading edge (Park et al., 2005; Sims et al., 2007; Stabile et al., 2010; Ishihara et al., 2012).

B. X-linked neutropenia

In contrast to loss-of-function mutations in WASP, three distinct mutations in the GTPase binding domain (L270P, S272P, and I294T) increase WASP’s actin polymerizing activity (Devriendt et al., 2001; Ancliff et al., 2006; Beel et al., 2009). These mutations disrupt the autoinhibitory interaction between the GTPase and VCA domains leading to unscheduled activation of Arp2/3. How excess actin polymerization induces severe congenital neutropenia is subject to current investigation. WASP I294T B and T cells exhibit signs of genome instability including elevated doublet and fused chromosomes and tetraploidy (Westerberg et al., 2010). However, the mechanism by which nuclear WASP might facilitate genomic transactions required for myelopoiesis is unknown.
Chapter 2: Manuscript

“Nuclear Arp2/3 drives DNA break clustering for homology-directed repair”

Sections of this chapter are published in *Nature* (Schrank et al., 2018). The formatting has been modified for the purpose of this thesis.
**TITLE:** Nuclear Arp2/3 drives DNA break clustering for homology-directed repair

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**AUTHOR CONTRIBUTIONS:**
J.G. and B.R.S conceived the study and wrote the manuscript. B.R.S. conducted the majority of the experiments and data analyses. T.A. and Y.L. performed the mass spectrometry experiments. G.G.G., M.E.G., W.C., B.T.C., T.A. and Y.L. assisted with data analysis and interpretation.

**ABSTRACT:**
DNA double-strand breaks repaired by non-homologous end joining display limited DNA end-processing and chromosomal mobility. By contrast, double-strand breaks undergoing homology-directed repair exhibit extensive processing and enhanced motion. The molecular basis of this movement is unknown. Here, using *Xenopus laevis* cell-free extracts and mammalian cells, we establish that nuclear actin, WASP, and the actin-nucleating ARP2/3 complex are recruited to damaged chromatin undergoing homology-directed repair. We demonstrate that nuclear actin polymerization is required for the migration of a subset of double-strand breaks into discrete sub-nuclear clusters. Actin-driven movements specifically affect double-strand breaks repaired by homology-directed repair in
G2 cell cycle phase; inhibition of actin nucleation impairs DNA end-processing and homology-directed repair. By contrast, ARP2/3 is not enriched at double-strand breaks repaired by non-homologous end joining and does not regulate non-homologous end joining. Our findings establish that nuclear actin-based mobility shapes chromatin organization by generating repair domains that are essential for homology-directed repair in eukaryotic cells.
INTRODUCTION:

DNA double-strand breaks (DSBs) induce chromatin movement. In budding yeast, which repair DSBs primarily by homology-directed repair (HDR), induction of a single chromosomal break triggers increased local mobility: the DSB mean-square displacement is substantially higher than that of an undamaged region (Dion et al., 2012; Miné-Hattab and Rothstein, 2012). Moreover, multiple DSBs form clusters after traversing long distances (Lisby et al., 2003). DSB clustering may facilitate homology search, increase repair efficiency or shield breaks from misrepair (Aten et al., 2004; Aymard et al., 2017). These movements are intricately related to HDR. Factors that are critical for initiation of resection and downstream recombination are essential for DSB mobility in yeast (Dion et al., 2012; Miné-Hattab and Rothstein, 2012). In mammalian cells, DSBs are often described as more stable, suggesting that non-homologous end joining (NHEJ), the predominant repair pathway, limits movement (Lemaitre et al., 2014; Robinett et al., 1996; Soutoglou et al., 2007). However, in human HeLa cells, RAD51-positive DSBs induced by alpha particles form clusters (Aten et al., 2004). Similarly, damaged telomeres in human U2OS cells that are maintained by recombination merge in a RAD51-dependent manner (Cho et al., 2014). Moreover, damaged active genes cluster in preparation for HDR (Aymard et al., 2017). Movement of deprotected mouse telomeres requires the LINC (linker of nucleoskeleton and cytoskeleton) complex, which transmits cytoskeletal forces
from the cytoplasm to the nucleus (Lottersberger et al., 2015). The molecular basis of DSB movement and its role in DNA repair remain unclear.

The machinery that drives actin polymerization in the cytoplasm is also found in the nucleus (Virtanen and Vartiainen, 2017). Specifically, the ARP2/3 complex and its activator WASP, a Wiskott–Aldrich Syndrome (WAS) protein family member, are located in both cellular compartments (Taylor et al., 2010; Wu et al., 2006; Yoo et al., 2007). WASP brings the ARP2 and ARP3 subunits into close proximity to activate the complex and enable filament elongation (Pollard and Borisy, 2003). Genotoxic agents trigger actin polymerization in the nucleoplasm of mammalian cells (Belin et al., 2015); however, the role of actin polymerization in DSB repair has not been characterized.

**RESULTS:**

**Actin nucleators bind damaged chromatin**

To better characterize the protein landscape at DNA double-strand breaks (DSBs), we performed an unbiased proteomics screen to document the recruitment of proteins to chromosomal DSBs in cell-free extracts derived from *Xenopus* eggs. PflMI restriction endonuclease was added to sperm chromatin in S-phase extracts to induce DSBs. Peptides derived from proteins enriched in the chromatin fraction were labeled with isobaric tags and subjected to liquid chromatography mass spectrometry. We then assessed the enrichment of peptides derived from DSB-containing chromatin relative to peptides obtained from chromatin purified from undamaged control samples. Validating our
approach, LC-MS/MS analyses revealed the enrichment of several known regulators of DSB repair, including the XRCC6/Ku80 subunit of the KU heterodimer and the Nbs1 subunit of the Mre11/Rad50/Nbs1 complex (Fig. 2-1a). In addition, we identified several proteins not previously known to be associated with the DNA damage response. Among such proteins were all seven subunits of the actin nucleating complex Arp2/3, as well as β-actin and actin filament capping proteins (Fig. 2-1a). We confirmed that β-actin, Arpc4, and CapZβ are recruited to Mre11-enriched, DSB-containing chromatin by Western blot (Fig. 2-1b). We next asked whether actin enrichment at chromosomal DSBs required DNA damage signaling. Inhibition of the phosphatidylinositol-3-OH kinase (PI(3)K)-like kinases ATM and ATR reduced the binding of actin complexes to damaged chromatin (Fig. 2-2a, b; Fig 2-3). Moreover, treatment with the small molecule inhibitor CK-666, which stabilizes the Arp2/3 complex in an open, inactive conformation (Hetrick et al. 2013; Nolen et al. 2009), significantly decreased Arpc4, β-actin, and CapZβ enrichment in damaged chromatin (Fig. 2-1b, c). CK-548, a different Arp2/3 inhibitor, which inserts into the hydrophobic core of the Arp3 subunit, also reduced accumulation of the Arp2/3 complex, β-actin, and CapZβ in damaged chromatin (Fig. 2-2c). Overall, these results reveal that PI3K-like kinases and the Arp2/3 complex regulate the assembly of actin polymerization machinery at chromosomal DSBs in *Xenopus* extracts.
Fig. 2-1 | Actin complexes are recruited to damaged chromatin.
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Identification of actin complexes and repair proteins in DSB-containing chromatin by liquid chromatography-mass spectrometry. The enrichment ratio and spectral counts are shown. 

b, Western blot showing enrichment of actin complexes in chromatin damaged using the PflMI restriction endonuclease (+PflMI). MRE11 indicates DNA damage. 

c, Protein quantification in chromatin relative to +PflMI samples. 

$P$ calculated by one-way ANOVA with multiple comparisons; data shown as mean and s.d.; $n = 5, 3, \text{ and } 4$ independent experiments, left to right.
Fig. 2-2 | Actin filament nucleators localize to chromosomal DSBs in Xenopus extracts and mammalian cells.
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*a*, DNA damage-dependent enrichment of actin complexes (+PfiMI) following PIKK inhibition (KU55933: ATMi; VE821: ATRi). RPA shown as a marker of DNA damage. 

*b*, Quantification of actin complexes in chromatin relative to +PfiMI samples. (*P* calculated by one-way ANOVA with multiple comparisons; data shown as mean and SD; β-actin *n* = 3, Arpc4 *n* = 2, CapZβ *n* = 3 independent experiments). 

*d*, Representative images of WASP foci after NCS treatment in MTFs. 

*e*, Quantification of WASP foci (*P* calculated by two-sided Mann–Whitney test; data shown as mean; DMSO *n* = 578 nuclei, NCS *n* = 556 nuclei). 

*f*, Co-localization of γH2AX with WASP foci in MTFs. *n* = 22 nuclei, *r* = 0.58 ± 0.021, Pearson. Scale bar, 5 μm or as indicated.
Fig. 2-3 | Source gels.
**Fig. 2-3 | Source gels.** This file contains source gels. **a,** Western blots showing recruitment of actin complexes and Mre11 to damaged chromatin, related to Fig. 2-1b. **b,** Western blots showing lack of Arpc2 protein in Arpc2-LoxP-CreER MTFs, related to Fig. 2-5j. **c,** Western blots showing expression of WT-NLS and R62D-NLS actin constructs in U2OS cells, related to Fig. 2-16e. **d,** Western blots showing recruitment of actin complexes and RPA to damaged chromatin, related to Fig. 2-2a. **e,** Western blots showing expression of DNA-PK, WASP, RPA, Rad51, and Arpc2 in U2OS cells, related to 2-8g. **f,** Western blots showing expression of WASP and N-WASP in U2OS cells, related to Fig. 2-16b. **g,** Western blots showing expression of WT, R62D, WT-NLS, and R62D-NLS actin tagged with mCherry in U2OS cells, related to Fig. 2-17a. **h,** Western blots showing expression of Formin 2 in U2OS cells, related to Fig. 2-16c.
**WASP and Arp2/3 localize to DSBs undergoing HDR**

We next tested whether WASP localized to DSB foci in mammalian cells. Generation of DSBs by neocarzinostatin (NCS), a radiomimetic antibiotic, induced the formation of WASP foci in U2OS cells (Fig. 2-4a, b). Moreover, WASP substantially co-localized with γH2AX, which marks large chromatin domains surrounding DSBs (Rogakou et al., 1999), suggesting that sites of DNA repair contain WASP (Fig. 2-4c). Similarly, WASP foci arose in mouse-tail fibroblasts (MTFs) after DSB generation and co-localized with γH2AX (Fig. 2-2d–f).

We next investigated whether actin complexes are recruited in close proximity to DSBs. Accordingly, we performed chromatin immunoprecipitation (ChIP) experiments in U2OS cells in which genome-wide DSBs had been generated by nuclear translocation of the AsiSI restriction enzyme (Aymard et al., 2014). We interrogated four DSB sites, annotated DSBs I–IV, in synchronized cells during the G1 or G2 phase of the cell cycle (Fig. 2-4d). First, we monitored the recruitment of RAD51 and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs). RAD51 has been shown to localize to a subset of these DSBs (Aymard et al., 2014). RAD51 bound exclusively to DSBs I and II, and this binding was more robust in the G2 phase than in the G1 phase (Fig. 2-4e). By contrast, DNA-PKcs was recruited to all four DSBs in both cell-cycle phases (Fig. 4-2f). Together, these results demonstrate that HDR can occur at a subset of DSBs in G2 and distinguished from DSBs undergoing NHEJ, the more frequent mode of repair.
We next tested whether actin filament nucleators localize to DSB sites. WASP accumulated at DSBs I and II in both cell cycle phases (Fig. 2-4g), whereas ARPC2 recruitment was restricted to G2 (Fig. 2-4h). Notably, WASP was also enriched at DSBs III and IV, whereas ARPC2 was not (Fig. 2-4g, h). Neither WASP nor ARPC2 were enriched at an undamaged site (Fig. 2-4i). Collectively, these data establish that WASP binds DSBs independently of repair pathway choice but specifically activates ARP2/3 at DSBs undergoing HDR. This supports a model in which actin polymerization is primed at all DSBs but is triggered only during the assembly of HDR machinery.
Fig. 2-4 | ARP2/3 and WASP co-localize at HDR breaks.
**Fig. 2-4 | ARP2/3 and WASP co-localize at HDR breaks.**  

**a,** Representative U2OS cells with WASP foci.  

**b,** Quantification of WASP foci.  

$P$ calculated by two-sided Mann–Whitney test; data shown as mean and s.d.; $n = 1,231$ (DMSO), 1,327 nuclei (NCS).  

**c,** Co-localization of $\gamma$H2AX with WASP ($n = 30$ nuclei; $r = 0.60 \pm 0.07$, Pearson).  

**d,** Cell cycle distribution of synchronized ER-AsiSI U2OS cells (U2OS cells stably expressing the AsiSI restriction endonuclease tethered to the oestrogen receptor) after thymidine release.  

**e–h,** ChIP showing enrichment of Rad51 (e), DNA-PKcs (f), WASP (g), and ARPC2 (h) at DSBs I–IV in G1 and G2 cells. Mean and s.d. ($n = 3$ technical replicates) of a representative experiment (out of two independent experiments). The cutting efficiency at each DSB site was previously published (Aymard et al., 2014).  

**i,** Mean and s.d. enrichment of ARPC2 and WASP at an undamaged site in G2 cells ($n = 3$ technical replicates) of a representative experiment (out of two independent experiments). Scale bar, 10 $\mu$m.
**Arp2/3 inactivation impairs DSB movement**

Increased chromatin mobility after DSB formation has been described in budding yeast and in mammalian cells (Aten et al., 2004; Neumaier et al., 2012; Miné-Hattab and Rothstein, 2012; Dion et al., 2012; Caron et al., 2015; Aymard et al., 2017; Smith et al., 2018). The ARP2/3 complex generates propulsive forces by nucleating a highly-branched network of actin filaments. As WASP and ARP2/3 localized to RAD51-bound DSBs, we investigated whether their activity induced movement and clustering of HDR machinery. Accordingly, we visualized DSB movements in a U2OS cell line that stably expresses RAD52 tagged with mCherry and 53BP1 tagged with yellow fluorescent protein (YFP) (Karanam et al., 2012). RAD52 mediates recombination and forms foci exclusively in S/G2 cells (Karanam et al., 2012; Ochs et al., 2016). Generation of DSBs in S/G2 cells yielded robust induction of RAD52–mCherry foci (Fig. 2-5a). The dynamic behavior of mCherry-RAD52 foci was evaluated by capturing confocal z stacks of selected nuclei every 5 min. Z stacks were subsequently projected onto a single plane in ImageJ and T stacks were aligned using the Stack-Reg plug-in to normalize for cell migration (Thevenaz et al., 1998). Strikingly, RAD52 foci clustered and merged over a 60-min interval (Fig. 2-5a, b). Coalescing foci increased in intensity and size and remained merged for a minimum of 15 min (Fig. 2-5b, c). Notably, inactivation of ARP2/3 using CK-666 decreased the frequency of clustering events and prevented the increase in the size of foci (Fig. 2-5c, d). We subsequently compared DSB movements in the presence of CK-666 with those in the presence of CK-689, an inactive control analogue (Nolen et
Quantitative analysis of DSB tracks in CK-689-treated cells indicated that RAD52 foci explored a mean cumulative distance of 4.1 \( \mu \)m over 100 min (Fig. 2-5e, f). The mean-square displacement (MSD) of RAD52 foci, which plots the average squared distance foci travel at increasing time intervals, as well as the diffusion coefficient \( D(t) \), which is approximated through the linear-weighted fit of the initial mean MSD curve, were subsequently determined (Tarantino et al., 2014). MSD analysis of RAD52 foci revealed a \( D(t) \) of \( 3.7 \times 10^{-5} \mu m^2 \) s\(^{-1} \) (Fig. 2-5g and Fig. 2-6a). Motion type analysis was consistent with confined Brownian motion. Critically, inhibition of ARP2/3 diminished the distance travelled by DSBs over the 100-min period (Fig. 2-5e, f). Furthermore, CK-666 treatment decreased the RAD52 diffusion coefficient from \( 3.7 \times 10^{-5} \mu m^2 \) s\(^{-1} \) to \( 1.8 \times 10^{-5} \mu m^2 \) s\(^{-1} \) (Fig. 2-5g).

We subsequently monitored DSBs bound by replication protein A (RPA) in MTFs expressing RPA32 tagged with enhanced GFP and a nuclear localization sequence (RPA32–pEGFP–NLS). Similar to the behaviour of RAD52–mCherry foci, RPA32 foci frequently merged over a 60-min interval (Fig. 2-5h). Notably, CK-666 treatment diminished the distance travelled by RPA foci relative to CK-689 treatment (Fig. 2-5i). Moreover, the diffusion coefficient calculated for RPA foci in cells treated with CK-666 decreased from \( 4.1 \times 10^{-5} \mu m^2 \) s\(^{-1} \) (for cells treated with CK-689) to \( 2.1 \times 10^{-5} \mu m^2 \) s\(^{-1} \) (Fig. 2-6a). Finally, we used MTFs harbouring a conditional Arpc2 allele (Rotty et al., 2015). Upon 4-OHT treatment, these cells are depleted of ARPC2\(^{23} \) (Fig. 2-5j). Consistent with CK-666-treated cells, RPA movements were significantly attenuated in ARCP2-depleted cells.
(Fig. 2-5i–l). CK-689 affected RAD52 and RPA foci movement only minimally relative to DMSO (Fig. 2-6a, b).

The formation of Rad51 filaments requires single-stranded DNA (ssDNA)–RPA intermediates. Thus, we assessed how ARP2/3 inactivation affected the behaviour of RAD51 foci. To circumvent the lack of a functional fluorescent RAD51 reporter, we used Icy bioimaging software and its spatial analysis plug-in (http://www.icy.bioimageanalysis.org) to assess clustering of RAD51 foci. This approach was validated in a study showing ATM-dependent clustering of γH2AX foci in fixed cells (Caron et al., 2015). Briefly, the spatial analysis plug-in utilizes Ripley’s K-function to assess the deviance of paired points from total randomness (Fig. 2-7a). When sufficient pairs of points congregate within a given radius, the software generates a K-function, which crosses the threshold of statistical significance indicating clustering events (Lagache, et al., 2013). Analysis with Icy software revealed that RAD51 foci were highly clustered in the nucleus and that CK-666 reduced this clustering (Fig. 2-7b–d). CK-666 treatment did not affect cell viability, cell cycle progression, nuclear size, nuclear morphology, or the expression of RPA or RAD51 (Fig. 2-8a–g). These experiments demonstrate that ARP2/3-mediated actin polymerization enhances DSB motion during HDR, thereby increasing the clustering of foci.

During the G1 phase of the cell cycle, 53BP1 forms foci and regulates end-joining events. Consistent with the lack of ARP2/3 at DSBs in G1 cells (Fig. 2-4), the movement of 53BP1–YFP foci in G1 cells was unaffected by ARP2/3 inhibition (Figs. 2-6a, 2-9a–c). In G2 cells, many 53BP1–YFP and RAD52 foci
colocalized, consistent with the localization of 53BP1 at sites of DSB resection\textsuperscript{22} (Fig. 2-9d). The diffusion coefficient for 53BP1 foci increased from \(2.4 \times 10^5\) \(\mu\text{m}^2\text{s}^{-1}\) in G1 cells to \(3.5 \times 10^5\) \(\mu\text{m}^2\text{s}^{-1}\) in G2 cells (Fig. 2-9e, f). Unlike in G1 cells, the motion of 53BP1 foci in G2 cells was reduced by ARP2/3 inactivation (Fig. 2-9g). Collectively, these data indicate that ARP2/3 enhances the motion of sites of DSB resection in G2 cells.

In mammalian cells, the majority of DSBs are repaired by NHEJ, not by HDR. \(\gamma\)H2AX marks all DSBs regardless of repair pathway choice and is present in the G1-, S- and G2-phases of the cell cycle. Given that NHEJ predominates in mammalian cells, the majority of \(\gamma\)H2AX-labelled DSBs are expected to resolve by NHEJ. The \(\gamma\)H2AX foci that remain are thought to reflect DSBs undergoing HDR: the slower mode of repair (Shibata et al., 2010). To test the effect of Arp2/3 activity on \(\gamma\)H2AX foci dynamics, we monitored the number and size of \(\gamma\)H2AX foci in fixed cells at different time points following DSB generation. \(\gamma\)H2AX foci were quantified after treating U2OS cells with NCS (Fig. 2-10a). Most NCS-induced DSBs resolved by 8 h following treatment (Fig. 2-10b, lanes 1 and 3). Importantly, the \(\gamma\)H2AX foci that remained at 8 hours were larger in size suggesting that the DSBs that remained had assembled into clusters (Fig. 2-10c). Notably, treatment with CK-666 or CK-548 resulted in persistent \(\gamma\)H2AX foci at 8 h, which were smaller in size (Fig. 2-10b, c and Fig. 2-11a). No significant difference in \(\gamma\)H2AX foci number or size were observed in cells treated with CK-666 2 h following DSB generation, indicating that the inhibitors did not compromise early repair events (Fig. 2-10b, c). Moreover, most \(\gamma\)H2AX foci
resolved by 8 h despite ARP2/3 inhibition indicating that the predominant repair pathway in cells, NHEJ, was intact (Fig. 2-10b, lanes 2 and 4).

To evaluate whether γH2AX foci merged into clusters between 2 h and 8 h post NCS treatment, we again employed the Icy bioimaging software and its spatial analysis plug-in. Following induction of DSBs with NCS, γH2AX foci clustered (Fig. 2-10d). However, this time-dependent clustering of γH2AX domains was abrogated following treatment with CK-666 or CK-548 (Fig. 2-10d; Fig. 2-11b). Similar findings were observed in mouse embryonic fibroblasts (MEFs) treated with NCS, suggesting that ARP2/3-mediated DSB clustering of γH2AX foci is a general feature of DNA repair (Fig. 2-11c-e). Finally, MTF cells depleted of ARPC2 exhibited persistent γH2AX foci 8 h post NCS exposure comparable to treatment with CK-666 (Fig. 2-10e-g) as well as defects in foci clustering (Fig. 2-10f). Taken together, these results indicate that the ARP2/3 complex is required for the clustering of DSBs with slower kinetics of repair.

Since WASP and ARP2/3 are both required to initiate actin nucleation, we wondered whether DSB mobility was also dependent on WASP activity. We treated U2OS cells with wiskostatin, a small molecule inhibitor that locks WASP in its autoinhibited conformation, thus abrogating activation of the ARP2/3 complex (Peterson et al., 2004). Following induction of DSBs with NCS, we noted significant persistence of γH2AX foci in wiskostatin-treated cells relative to controls, and no apparent clustering of DSBs (Fig. 2-10h, i). Since wiskostatin also interacts with and inhibits the WASP cytoplasmic homolog N-WASP, we specifically depleted cells of WASP by RNA interference (Fig. 2-10j). Comparable
defects in DSB clustering were observed (Fig. 2-10k). These findings were further validated in NCS-treated MEFs (Fig. 2-11f, g). The level of wiskostatin used did not affect cell viability or cell cycle progression (Fig. 2-11h, i). Collectively, these data indicate that WASP promotes DSB clustering by activating the ARP2/3 complex.
Fig. 2-5 | Arp2/3 drives DSB mobility during HDR.
Fig. 2-5 | Arp2/3 drives DSB mobility during HDR. a, A representative U2OS cell nucleus with boxes indicating clustering of RAD52–mCherry foci. b, Expanded images of clustering from a (red box). Circles denote intensity of foci. c, Size of RAD52 foci (P calculated by one-way ANOVA with multiple comparisons; data shown as mean and s.e.m.; n = 19 nuclei (DMSO), 17 nuclei (CK-666). d, RAD52–mCherry clustering events (P calculated by two-tailed Mann–Whitney test; data shown as mean and s.e.m.; n = 17 nuclei (DMSO), 16 nuclei (CK-666). e, Traces of RAD52–mCherry foci over 100 min. Expanded images of boxed tracks (right). f, Median cumulative distance travelled by RAD52–mCherry foci. P calculated by two-tailed Mann–Whitney test; n = 1,120 foci from 13 nuclei (CK-689), 720 foci from 12 nuclei (CK-666). g, MSD of RAD52–mCherry foci. Data shown as mean and weighted s.e.m.; n = 3,262 foci from 13 nuclei (CK-689), 2,143 foci from 12 nuclei (CK-666). Δt, time interval. h, Clustering of pEGFP–RPA32–NLS foci in MTFs. i, Traces of RPA32–pEGFP–NLS foci over 100 min. j, ARPC2 protein levels in whole-cell lysates from Arpc2-LoxP-CreER MTFs (single experiment). k, Median cumulative distance travelled by RPA32–pEGFP–NLS foci. P calculated by one-way ANOVA with multiple comparisons; n = 381 foci from 13 nuclei (CK-689), 370 foci from 12 nuclei (CK-666), 425 from 13 nuclei (4-OHT). l, MSD of RPA32–pEGFP–NLS foci. Data shown as mean and weighted s.e.m.; n = 790 foci from 13 nuclei (CK-689), 823 foci from 12 nuclei (CK-666), 1135 foci from 13 nuclei (4-OHT). Scale bars, 5 µm or as indicated.
Fig. 2-6 | CK-689 does not significantly alter movement of foci.
Fig. 2-6 | CK-689 does not significantly alter movement of foci. a, Summary of DSB movement analyses as related to Figs. 2-5, 2-9, and 2-18. b, MSD of RAD52–mCherry foci and RPA–pEGFP–NLS foci treated with DMSO, CK-689 and CK-666. Data shown as mean and weighted s.e.m.; RAD52: CK-689

$n = 3,262$ foci from 13 nuclei, CK-666 $n = 2,143$ foci from 12 nuclei, DMSO $n = 3,292$ foci from 12 nuclei; RPA32: CK-689 $n = 790$ foci from 13 nuclei, CK-666 $n = 823$ foci from 12 nuclei, DMSO $n = 1,031$ foci from 10 nuclei. MSD of RAD52 DMSO also shown in Fig. 2-18i. MSD of RAD52 CK-689 and CK-666 also shown in Fig. 2-5g. MSD of RPA CK-689 and CK-666 also shown in Fig. 2-5i.
Fig. 2-7 | ARP2/3 clusters RAD51 foci.
Fig. 2-7 | ARP2/3 clusters RAD51 foci. a, Schematic of clustering events, as defined by the number of paired points that fall within a given radius (arrow, dashed circle). b, Representative cell cycle distribution of U2OS cells following double-thymidine block (out of five independent experiments). c, Representative images of U2OS-AsiSI nuclei showing RAD51 foci. d, RAD51 foci clustering in G2 cells ($P$ calculated by Spatial analysis plug-in; data shown as mean and s.e.m. of Ripley function; DMSO $n = 95$ nuclei, CK-666 $n = 80$ nuclei).
Fig. 2-8 | WASP and ARP2/3 inactivation do not impair U2OS cell viability, nuclear area, nuclear sphericity or protein expression.
Fig. 2-8 | WASP and ARP2/3 inactivation do not impair U2OS cell viability, nuclear area, nuclear sphericity or protein expression. a, Cell survival after 8 h of treatment with 100 µM CK-666, 50 µM CK-548, or 3 µM wiskostatin. Data shown as mean and s.e.m. n = 4 independent experiments. b, Cell survival after 48 h exposure to increasing concentrations of CK-666. n = 3 independent experiments. Data shown as mean and s.e.m. c, Cell survival after 48 h exposure to increasing concentrations of CK-548. n = 3 independent experiments. Data shown as mean and s.e.m. d, Representative cell-cycle distribution of cells treated with DMSO, 50 µM CK-666, or 25 µM CK-548 for 48 h (out of three independent experiments). e, Analysis of nuclear sphericity. P calculated by Student’s two-tailed t-test; data shown as mean and s.d.; DMSO n = 117 nuclei, CK-666 n = 117 nuclei. NS, not significant. f, Analysis of nuclear area. P calculated by one-way ANOVA with multiple comparisons; data shown as mean and s.d.; DMSO n = 210 nuclei, CK-548 n = 189 nuclei, CK-666 n = 92 nuclei. g, Expression of RAD51, DNA-PKcs, WASP, and ARPC2 validating antibodies used in Fig. 2-4e-i (single experiment). MEK2 is a loading control. RPA expression levels also shown.
Fig. 2-9 | ARP2/3 enhances movement of 53BP1 foci in G2 cells.
Fig. 2-9 | ARP2/3 enhances movement of 53BP1 foci in G2 cells. a, Representative U2OS nuclei showing 53BP1–YFP focus traces over 100 min in G1 cells. b, Median cumulative distance travelled by 53BP1–YFP foci in G1 cells. $P$ calculated by two-tailed Mann–Whitney test; CK-689 $n = 462$ foci from 14 nuclei, CK-666 $n = 647$ foci from 14 nuclei. NS, not significant. c, MSD of 53BP1–YFP foci in G1 cells. Data shown as mean and weighted s.e.m.; CK-689 $n = 926$ foci from 14 nuclei, CK-666 $n = 1,234$ foci from 14 nuclei. $\Delta t$, time interval. d, Left, a representative G1 cell with 53BP1–YFP foci. Right, a representative G2 cell with 53BP1–YFP and Rad52–mCherry foci. 53BP1 foci colocalize with RAD52 foci. $r = 0.41 \pm 0.17$, Pearson, $n = 5$ independent experiments. e, Representative U2OS nuclei showing traces of 53BP1–YFP foci over 100 min in G2 cells. f, MSD of 53BP1–YFP foci. Data shown as mean and weighted s.e.m.; G1 CK-689 $n = 926$ foci from 14 nuclei, G2 CK-689 $n = 1,403$ foci from 12 nuclei. G1 CK-689 curve also shown in c. g, MSD of 53BP1–YFP foci in G2 cells. Data shown as mean and weighted s.e.m.; CK-689 $n = 1,403$ foci from 12 nuclei, CK-666 $n = 1,038$ foci from 10 nuclei. G2 CK-689 curve also shown in f.
Fig. 2-10 | ARP2/3 clusters DSBs with slow kinetics of repair.
Fig. 2-10 | ARP2/3 clusters DSBs with slow kinetics of repair. a,
Representative images of γH2AX foci in U2OS cells. b, γH2AX foci are
enumerated 2 and 8 h after application of NCS followed by DMSO or 100 μM CK-666. (At 2 h, n > 750 nuclei per condition analyzed in two independent
experiments. At 8 h, n > 7000 nuclei per condition analyzed in four independent experiments). Red line indicates median number of foci per cell. p-values are
from two-tailed Mann-Whitney test. c, γH2AX foci size was quantified by the Icy
software Spot Detector plug-in. Columns represent mean and s.e.m. of γH2AX
foci size in cells treated with DMSO or CK-666 (n > 100 nuclei per condition
analyzed in three independent experiments). One-way ANOVA with multiple
comparisons was used to determine p-values. d, γH2AX foci clustering is
modeled by Ripley’s K function, which tests the spatial randomness of observed
spots. Clustering of NCS-induced γH2AX foci was determined by the Icy software
spatial analysis plug-in, which reports statistically significant clustering when the
Ripley function crosses the clustering threshold (p=0.05). Clustering events are
assessed 2 and 8 h after NCS in cells treated with DMSO (left) or 100 μM CK-666 (right). (At 2 h, n > 150 nuclei per condition analyzed in two independent
experiments. At 8 h, n > 100 nuclei per condition in three independent
experiments). Curves represent mean and s.e.m of Ripley function. e, NCS-
induced γH2AX foci arise 8 h after application of NCS in MTF wild-type and
Arpc2/- fibroblasts. f, Clustering of NCS-induced γH2AX foci are assessed in
MTF wild-type and Arpc2/- fibroblasts (n > 50 nuclei per condition in two
independent experiments). g, γH2AX foci enumeration in wild-type and Arpc2/-
MTFs treated with DMSO or 100 μM CK-666 (n > 100 nuclei per condition in two independent experiments). One-way ANOVA with multiple comparisons was used to determine p-values. h, U2OS cells were treated for 1 h with NCS, washed, then incubated in DMSO or 3 μM wiskostatin (W.S.). At 8 h post NCS, cells were washed, fixed, and prepared for γH2AX foci enumeration. At 8 h, n > 1000 nuclei per condition analyzed in three independent experiments. Red line indicates median number of foci per cell. p-values are from two-tailed Mann-Whitney test. i, Clustering of γH2AX foci in the presence of DMSO or 3 μM wiskostatin (n > 100 cells per condition in 2 independent experiments). Curves represent mean and s.e.m of Ripley function. j, U2OS cells were transfected with siRNA directed against the WASP protein or a mock siRNA for 24 h. Western blot shows expression of WASP and N-WASP following siRNA knockdown in whole cell lysates. k, NCS-induced clustering of γH2AX foci following knockdown with WASP siRNA or mock siRNA (n > 80 cells per condition). Curves represent mean and s.e.m of Ripley function.
Fig. 2-11 | WASP and ARP2/3 cluster γH2AX foci in U2OS and MEFs.
Fig. 2-11 | WASP and ARP2/3 cluster γH2AX foci in U2OS and MEFs. a, U2OS cells were treated for 1 h with NCS, washed, then incubated in DMSO or 50 μM CK-548. At 8 h post NCS, cells were washed, fixed, and prepared for γH2AX foci enumeration. At 8 h, n > 1700 nuclei per condition analyzed in four independent experiments. Red line indicates median number of foci per cell. p-values are from two-tailed Mann-Whitney test. b, Clustering of NCS-induced γH2AX foci was determined by the Icy software spatial analysis plug-in, which reports statistically significant clustering when the Ripley function crosses the clustering threshold. Clustering events are assessed 8 h after NCS in cells treated with 50 μM CK-548. (n > 150 nuclei per condition analyzed in four independent experiments). c, NCS induced DSBs in mouse embryonic fibroblasts (MEFs), detected by γH2AX foci. d, γH2AX foci are enumerated 2 and 8 h after application of NCS in MEFs, in the presence of DMSO or 100 μM CK-666. (At 2 h, n > 700 nuclei per condition analyzed in two independent experiments. At 8 h, n > 500 nuclei per condition analyzed in two independent experiments. p-values are from two-tailed Mann-Whitney test. e, Clustering of NCS-induced γH2AX foci was determined by the Icy software spatial analysis plug-in, which reports statistically significant clustering when the Ripley function crosses the clustering threshold. Clustering events are assessed 2 and 8 h after NCS in MEFs treated with DMSO or 100 μM CK-666. (At 2 h, n > 90 nuclei per condition analyzed in two independent experiments. At 8 h, n > 90 nuclei per condition in two independent experiments). Curves represent mean and s.e.m of Ripley function. f, γH2AX foci are enumerated 2 and 8 h after application of NCS
in MEFs, in the presence of DMSO or 3 μM wiskostatin (At 2h, n > 750 nuclei per condition analyzed in two independent experiments. At 8 h, n > 500 nuclei per condition analyzed in two independent experiments. p-values are from two-tailed Mann-Whitney test. g, Clustering of NCS-induced γH2AX foci are assessed 8 h after NCS in MEFs treated with DMSO or 3 μM wiskostatin. (At 8 h, n > 35 nuclei per condition). Curves represent mean and s.e.m of Ripley function (**p<0.01). h, U2OS cells were bathed in media supplemented with serum, antibiotics, and wiskostatin. U2OS cell viability was assessed 48 h after the application of the compound. i, Fluorescence-activated cell sorting was used to determine the cell-cycle distribution of cells treated with DMSO or 3 μM wiskostatin for 48 h.
ARP2/3 nucleates actin foci at sites of HDR

Genotoxic agents induce nuclear actin structures in mammalian cells (Belin et al., 2015). In U2OS cells transfected with nuclear actin-chromobody TagGFP (Plessner et al., 2015), we identified a subset of actin structures resembling foci (actin-cb foci); the number of these foci increased after NCS treatment (Fig. 2-12a, b and Fig. 2-13a, b). We also identified brighter, rod-like filaments (Fig. 2-13a, b). Strikingly, actin-cb foci clustered and merged over a 20-min interval (Fig. 2-12c). MSD analysis of actin-cb foci revealed a $D(t)$ of $3.4 \times 10^{-4} \, \mu m^2 \, s^{-1}$ (Fig. 2-12d, e). Similar to RAD52 and RPA, the $\alpha$-coefficient for actin-cb foci indicated confined motion. Notably, CK-666 abolished actin-cb foci, strongly suggesting that these structures are sites of ARP2/3-dependent nucleation (Fig. 2-12f, g and Fig. 2-13d). By contrast, nuclear actin rods exhibited limited mobility and were unaffected by CK-666 (Fig. 2-13c–d). Finally, we compared the localization of actin-cb foci with RAD51 foci in fixed cells and RPA32–mCherry foci using live cell imaging. Following NCS treatment, 46% of RAD51 foci overlapped or abutted actin-cb foci (Fig. 2-12h). Moreover, we observed co-localization of actin-cb foci and RPA32–mCherry foci, which travelled together in the nucleoplasm (Fig. 2-12i and Fig. 2-13e, f). Collectively, these experiments provide direct evidence that ARP2/3 assembles dynamic nuclear actin structures at sites of HDR.
Fig. 2-12 | Nuclear actin foci cluster and localize to HDR sites.
Fig. 2-12 | Nuclear actin foci cluster and localize to HDR sites. a, Representative images of U2OS nuclei transfected with nuclear actin-chromobody TagGFP. Boxes indicate clustering events. b, Percentage of cells with actin-cb foci. *P* calculated by Student’s two-tailed *t*-test; data shown as mean and s.d.; *n* = 473 nuclei (DMSO), 473 nuclei (NCS). c, Expanded images of a clustering event from a (red box). Circles denote intensity of foci. d, Representative traces of actin-cb foci (out of three independent experiments) over 10 min. e, MSD of actin-cb foci. Data shown as mean and weighted s.e.m.; DMSO *n* = 662 foci from 11 nuclei. f, Representative images of a U2OS nucleus with actin-cb foci following CK-666 treatment. g, Quantification of actin-cb foci before and after CK-666 treatment. *P* calculated by paired two-tailed *t*-test; data shown as mean and s.e.m.; *n* = 10 nuclei from two independent experiments. h, Representative images of a U2OS nucleus showing RAD51 co-localization with actin-cb foci. The percentage of RAD51 foci that overlap or touch actin-cb foci is shown (*n* = 12 nuclei). i, Representative images of a U2OS nucleus (from five independent experiments) showing traces of RPA32–mCherry–NLS foci (red) and actin-cb foci (green). Scale bars, 5 µm or as indicated.
Fig. 2-13: Arp2/3 promotes actin foci assembly following DNA damage.
Fig. 2-13: Arp2/3 promotes actin foci assembly following DNA damage. a, Representative U2OS nuclei showing classes of nuclear actin structures following transient transfection of nuclear actin-chromobody-tag-GFP. b, Percentage of cells with diffuse signal, nuclear actin-cb foci, or rods with or without NCS (DMSO n=473 cells, NCS n=473 cells). c, MSD of actin-cb foci and actin-cb rods (Data shown as mean and weighted s.e.m.; actin-cb foci n=662 foci from 11 nuclei, actin-cb rods n=161 rods from 5 nuclei). d, Representative images of U2OS nuclei with actin rods or actin-cb foci following CK-666 treatment. e, Representative image of a U2OS nucleus with RPA-mCherry and actin-cb foci. Arrowheads indicate sites of RPA-mCherry and actin-cb co-localization. f, Expanded image of RPA-mCherry and actin-cb co-localization from e, red box. Traces of RPA and actin-cb foci are shown in red. Yellow and purple circles encompass RPA and actin-cb foci, respectively.
**WASP and ARP2/3 mediate HDR but not NHEJ**

To determine how DSB clustering affects DNA repair pathways, we used a panel of U2OS cell lines to monitor the repair of DSBs induced by the yeast endonuclease I-SceI by HDR, NHEJ, single-strand annealing (SSA), and microhomology-mediated end joining (MMEJ) (Gunn and Stark, 2012).

Inactivation of ARP2/3 by CK-666 or CK-548 reduced the ability of cells to repair I-SceI-induced DSBs using HDR or SSA by 40% (Fig. 2-14a, b, Fig. 2-15a, b, and 2-16a, e). Similarly, wiskostatin reduced the efficiency of HDR and SSA (Fig. 2-15a, b). Cells depleted of WASP by RNA interference showed similar defects in HDR (Fig. 2-16b–d). Notably, inhibition of WASP or ARP2/3 did not compromise MMEJ or NHEJ, neither of which require substantial resection (Fig. 2-14c, d, Fig. 2-15c, d, and Fig. 2-16f, g). Finally, the application of CK-689 did not reduce the repair of I-SceI-induced DSBs by HDR, SSA or NHEJ (Fig. 2-16h).

Although the ARP2/3 complex is found in the nucleus (Yoo et al., 2007), it also polymerizes actin in the cytoplasm. To support the idea that the HDR defect was a direct consequence of inhibiting nuclear actin polymerization, we increased nuclear actin levels by overexpressing actin fused to a nuclear localization sequence (actin–NLS) (Fig. 2-15e). Whereas expression of wild-type actin–NLS did not affect HDR, overexpression of an actin variant that interrupts filament formation (actin(R62D)–NLS) inhibited HDR to levels comparable with ARP2/3 inhibition (Fig. 2-15f). Treatment of cells expressing actin(R62D)–NLS with CK-666 did not further reduce HDR, suggesting that nuclear actin and ARP2/3 functioned together. Moreover, expression of wild-type actin,
actin(R62D), or wild-type actin–NLS tagged with mCherry did not affect DSB repair, whereas expression of actin(R62D)–NLS significantly inhibited HDR (Fig. 2-17a, b). This strongly suggests that inhibition of actin polymerization in the cytoplasm does not influence DNA repair in the nucleus. Similar to CK-666, expression of actin(R62D)–NLS did not reduce NHEJ-based repair (Fig. 2-15g). Finally, neither small inhibitory RNA (siRNA) directed against the actin filament nucleator formin-2 nor treatment with the formin inhibitor SMIFH2 inhibited HDR efficiency (Fig. 2-17c–f). This observation demonstrates a specific requirement for ARP2/3 complex activity in HDR.
Fig. 2-14 | GFP-based reporter assays for DSB repair.
Fig. 2-14 | GFP-based reporter assays for DSB repair. U2OS cell lines harbor specific GFP-based reporters to monitor distinct repair events (Gunn and Stark, 2012). These well-characterized assays respectively monitor the repair of I-Scel-induced DSBs by homology-directed repair (a), single-strand annealing (b), non-homologous end joining (c) and microhomology-mediated end joining (d). Notably, restoration of the intact GFP gene following DSB induction requires 2.7 kb of resection in the SSA (SA-GFP) reporter system, 464 bp of resection in the HRD (DR-GFP) reporter system, and 8 bp of resection in the Alt-EJ (EJ2-GFP) reporter system. GFP+ cells are monitored by FACS 48 h following transfection of I-Scel.
Fig. 2-15 | WASP and ARP2/3 mediate HDR.
Fig. 2-15 | WASP and ARP2/3 mediate HDR. a, Summary of DR-GFP assay, which monitors recombination between two GFP repeats (n = 10, 6, 4, 4 left to right). NS, not significant. b, Summary of SA-GFP (SSA) assay (n = 7, 4, 4, 3 left to right). c, Summary of EJ2-GFP (MMEJ) assay (n = 4, 3, 3, 3, left to right). d, Summary of EJ5-GFP (NHEJ) assay (n = 14, 6, 6, 3, left to right). e, Western blot shows FLAG-WT-NLS or FLAG-R62D-NLS overexpression in U2OS nuclear soluble fraction (single experiment). f, DR-GFP assay in cells transfected with FLAG-WT-NLS or FLAG-R62D-NLS. (n = 7, 7, 7, left to right). HDR efficiency in the presence of DMSO or CK-666 (Fig. 5a) shown for comparison. g, EJ5-GFP assay in cells transfected with FLAG-WT-NLS or FLAG-R62D-NLS. (n = 6, 6, 6, left to right). For 6a-g, P calculated by one-way ANOVA with multiple comparisons as indicated; data shown as mean and s.e.m. n represents independent experiments. WS, Wiskostatin.
Fig. 2-16: WASP and Arp2/3 mediate DSB repair by homology-directed mechanisms.
Fig. 2-16: WASP and Arp2/3 mediate DSB repair by homology-directed mechanisms. a, Representative FACS plots of GFP⁺ cells in the HDR (DR-GFP) assay. b, Western blot shows expression of WASP and N-WASP following siRNA knockdown in whole cell lysates (WASP siRNA 1: 5' GAGUGGCUGAGUUACUUGC 3'). c, Representative FACS plots of GFP⁺ cells in the HDR (DR-GFP) assay in WASP and mock-depleted cells. d, Summary of DR-GFP assay with WASP depletion (P calculated by one-way ANOVA with multiple comparisons; data shown as mean and s.e.m; n=3). e, Representative FACS plots of GFP⁺ cells in the SSA (SA-GFP) assay. f, Representative FACS plots of GFP⁺ cells in the MMEJ (EJ2-GFP) assay. g, Representative FACS plots of GFP⁺ cells in the NHEJ (EJ5-GFP) assay. h, Summary of DR-GFP, SA-GFP, and EJ5-GFP assays with CK-689 (P calculated by two-way ANOVA with multiple comparisons; data shown as mean and s.d.; n=3). n.s = not significant.
Fig. 2-17: Actin nucleation regulates HDR in the nucleus and does not require formin-2 activity.
Fig. 2-17: Actin nucleation regulates HDR in the nucleus and does not require formin-2 activity. a, Western blot shows expression of mCherry-tagged actin constructs in U2OS whole cell lysates. b, Summary of DR-GFP (HDR) assay ($P$ calculated by one-way ANOVA with multiple comparisons; data shown as mean and s.e.m; n=6). c, Western blot shows knockdown of formin-2 following 48 h transfection of mock or sequence-specific siRNA oligos. (Formin-2 siRNA #2: 5’-CGUGUAUCAGAAUGCCCA-3’). d, Summary of DR-GFP assay ($P$ calculated by one-way ANOVA with multiple comparisons; data shown as mean and s.e.m; n=3). e, Cell survival after 48 hours of increasing concentrations of the formin inhibitor, SMIFH2 (n=3 independent experiments). f, Summary of DR-GFP assay ($P$ calculated by student’s two-tailed t-test; data shown as mean and s.e.m; n=3).
ARP2/3 activity enhances DSB resection

To assess the functional importance of ARP2/3-driven DSB mobility, we compared the repair kinetics of two AsiSI-induced DSB sites (DSBs V and VI) that undergo up to 3.5 kb of resection (Zhou et al., 2014). In G1 cells, the repair kinetics of both DSBs were identical in the presence or absence of CK-666, confirming that ARP2/3 does not influence NHEJ (Fig. 2-18a). By contrast, in G2 cells, CK-666 substantially slowed repair at these DSB sites (Fig. 2-18b).

To investigate how actin-driven clustering might promote HDR efficiency, we tested whether the ARP2/3 complex facilitates resection. Thus, we directly measured the amount of single-stranded DNA (ssDNA) at DSBs V and VI that were recently shown to undergo up to 3.5 kb of resection downstream of the breaks (Zhou and Paull, 2015). Briefly, genomic DNA was extracted from AsiSI cells after treatment with 4-OHT and digested with a restriction endonuclease that cleaves double-stranded DNA but leaves ssDNA intact. Enrichment of ssDNA was quantified at three sites distal to DSBs by quantitative PCR in untreated cells and in cells treated with CK-666 and CK-548 inhibitors. Inactivation of the ARP2/3 complex decreased ssDNA generation at DSBs V and VI by 50%, indicating that clustering facilitates DSB end-resection (Fig. 2-18c). We consolidated this finding by quantifying RAD51 foci that arose following DSB resection in NCS-treated cells over 24 h (Fig. 2-18d, e). In control cells, the number of RAD51 foci first increased between 2 and 8 h and then decreased up to 24 h, indicative of recruitment and repair (Fig. 2-18d). By contrast, RAD51 foci increased minimally following ARP2/3 inhibition (Fig. 2-18d, e). Moreover,
substantially fewer RAD51 foci arose in ARPC2-depleted MTFs than in control MTFs (Fig. 2-18f, g).

In yeast and human cells, DSB motion requires the HDR machinery (Dion et al., 2012; Miné-Hattab and Rothstein, 2012; Aymard et al., 2017). We wondered whether RAD52 movement requires the activity of the MRN (MRE11, RAD50 and NBS1) complex (Symington and Gautier, 2011). Accordingly, we treated U2OS cells with mirin, a small-molecule inhibitor that abolishes the nuclease activity of MRE11 (Dupre et al., 2008). Mirin lowered ssDNA levels at DSBs V and VI by 70% (Fig. 2-18h). Notably, mirin substantially decreased the $D(t)$ of RAD52 foci from $4.4 \times 10^{-5} \mu m^2 s^{-1}$ to $2.4 \times 10^{-5} \mu m^2 s^{-1}$ (Fig. 2-18i and Fig. 2-6a) but did not significantly affect the MSD of 53BP1 foci in G1 cells (Fig. 2-18j). Together, these observations suggest a positive feedback loop, wherein resection enhances DSB movements, which, in turn, enhance resection.

WAS is an X-linked disorder characterized by severe immunodeficiency and predisposition to non-Hodgkin’s lymphoma and leukaemia (Masaad et al., 2013). Given that inactivation of WASP by wiskostatin or siRNA produced defects in HDR (Fig. 2-15a and Fig. 2-16d), we tested B lymphocytes derived from patients with WAS for defects in DSB end-resection. Relative to lymphocytes derived from healthy individuals, lymphocytes bearing two distinct WAS mutations exhibited 50% fewer RPA-positive cells upon exposure to the DNA topoisomerase I inhibitor camptothecin (CPT), consistent with a resection defect (Fig. 2-18k and Fig. 2-19a–d). Moreover, WAS mutant cells were more sensitive to CPT treatment than healthy lymphocytes (Fig. 2-20a, b). Inhibition of
ARP2/3 by CK-666 also enhanced CPT-induced lethality in U2OS cells (Fig. 2-20c). Finally, inhibition of ARP2/3 sensitized cells to the DNA polymerase inhibitor aphidicolin and the PARP1 inhibitor olaparib, indicating a reduced tolerance to replication stress (Fig. 2-20d, e). Together, these results demonstrate that activation of ARP2/3 by WASP enhances the processing and resolution of DSBs undergoing HDR.
Fig. 2-18 | ARP2/3 facilitates resection and repair in G2.
Fig. 2-18 | ARP2/3 facilitates resection and repair in G2. a, DSB repair at DSBs V and VI in G1-synchronized, ER-AsiSI-AID U2OS cells. Mean and s.d. ($n = 3$ technical replicates) of a representative experiment shown. b, DSB repair at DSBs V and VI in G2-synchronized ER-AsiSI-AID U2OS cells. Mean and s.d. ($n = 3$ technical replicates) of a representative experiment shown. c, Enrichment of ssDNA at DSBs V and VI following CK-548 treatment. Data shown as mean and s.d., $n = 6$ replicates from two independent experiments. d, Representative images of U2OS nuclei showing RAD51 foci. e, Quantification of RAD51 foci. $P$ calculated by one-way ANOVA with multiple comparisons; smoothed traces show distribution of RAD51 foci. Arrows above curves indicate mean number of foci per cell. DMSO: 2 h $n = 1,830$ cells, 8 h $n = 2,440$ cells, 24 h $n = 782$ cells; CK-666: 2 h $n = 2,438$ cells, 8 h $n = 2,189$ cells, 24 h $n = 912$ cells. f, Representative images of ARPC2-LoxP-CreER MTF nuclei showing RAD51 foci. g, Quantification of RAD51 foci in MTFs. $P$ calculated by two-tailed Mann–Whitney test; data shown as mean; $n = 571$ nuclei (DMSO), 462 nuclei (4-OHT). h, Representative ssDNA enrichment at DSBs V and VI following mirin treatment. Data shown as mean and s.d.; $n = 3$ replicates of two independent experiments. ssDNA in DMSO-treated cells (Fig. 6c) shown for comparison. i, MSD of RAD52–mCherry foci. Data shown as mean and weighted s.e.m.; DMSO $n = 3,292$ foci from 12 cells, mirin $n = 2,677$ foci from 11 cells. j, MSD of 53BP1–YFP foci in G1 cells. Data shown as mean and weighted s.e.m.; DMSO $n = 893$ foci from 12 cells, mirin $n = 744$ foci from 14 cells. k, Quantification of chromatin-bound RPA in S-phase cells. $P$ calculated by two-way ANOVA with multiple comparisons;
data shown as mean and s.e.m.; $n = 4$ independent experiments. WAS IVS6+5G>A and WAS V75M are cells carrying WAS mutations. RD and CB33, healthy lymphocytes. All DMSO columns, not significant ($P = 0.1789$). For CPT, CB33 versus RD, not significant ($P = 0.9643$); WAS IVS6+5G>A versus WAS V75M, not significant ($P = 0.9879$); CB33 versus WAS IVS6+5G>A, $P = 0.0045$; CB33 versus WAS V75M, $P = 0.0023$; RD versus WAS IVS6+5G>A, $P = 0.0016$; RD versus WAS V75M, $P = 0.0008$. 
Fig. 2-19: B-lymphocytes derived from Wiskott-Aldrich Syndrome patients exhibit reduced DSB end-resection.
**Fig. 2-19: B-lymphocytes derived from Wiskott-Aldrich Syndrome patients exhibit reduced DSB end-resection.**

a, The cell cycle distribution of CB33 and RD lymphocytes derived from healthy controls following DMSO treatment is shown (left). The percentage RPA positive S-phase cells following DMSO treatment was measured by flow cytometry (right).

b, The cell cycle distribution of lymphocytes bearing the V75M mutation in the WAS gene or a G>A transition at position 5 in intron 6 of the WAS gene (IVS6+5G>A) is shown following DMSO treatment (left). The percentage RPA positive S-phase cells following DMSO treatment was measured by flow cytometry (right).

c, The cell cycle distribution of CB33 and RD lymphocytes derived from healthy controls following CPT treatment (left). The percentage RPA positive S-phase cells following CPT treatment was measured by flow cytometry (right).

d, The cell cycle distribution of V75M or IVS6+5G>A lymphocytes following CPT treatment is shown (left). The percentage RPA positive S-phase cells following CPT treatment was measured by flow cytometry (right).
Fig. 2-20: Arp2/3 inactivation confers sensitivity to DSBs induced in S-phase as well as replication stress-inducing agents.
Fig. 2-20: Arp2/3 inactivation confers sensitivity to DSBs induced in S-phase as well as replication stress-inducing agents. a, Control CB33 lymphocytes or lymphocytes bearing a V75M mutation in the WAS gene were treated with camptothecin (CPT) for 0, 12, or 24 hours. Percent viability following CPT treatment was assessed by measuring the fraction of Annexin V and propidium iodide negative cells by flow cytometry. b, Summary of CB33 or WAS V75M lymphocyte survival following CPT treatment (P calculated by two-way ANOVA with multiple comparisons; data shown as mean and s.d.; n=3) c, Clonogenic U2OS cell survival after 12 h of camptothecin treatment in the presence of DMSO or increasing concentrations of CK-666. (Triplicate experiments; data shown as mean and s.d; n=2). d, Clonogenic U2OS cell survival after 12 h of aphidicolin treatment in the presence of DMSO or increasing concentrations of CK-666. (Triplicate experiments; data shown as mean and s.d; n=2). e, Clonogenic U2OS cell survival after olaparib treatment in the presence of DMSO or increasing concentrations of CK-666 for 14 days. (Triplicate experiments; data shown as mean and s.d; n=2). f, DNA damage induces DSBs, which are repaired preferentially by NHEJ in mammalian cells (in blue). In S/G2, DSBs may be repaired by HDR (in red). All DSBs recruit WASP, but Arp2/3-dependent actin polymerization occurs only at HDR breaks, which become more mobile. Actin polymerization in the vicinity of DSBs generates forces resulting in DSB clustering, optimal DNA end resection, Rad51 foci formation, and HDR.
DISCUSSION

DSB mobility is influenced by cell cycle stage, chromatin state, and repair pathway choice. Our finding that ARP2/3 specifically enhances the movement of breaks undergoing HDR is supported by studies showing that DSB mobility requires DNA end-resection. DSBs induced in pericentric heterochromatin during the S and G2 phases of the cell cycle relocate to the nuclear periphery, whereas DSBs generated during the G1 phase remain stable (Tsouroula et al., 2016). Critically, blocking HDR or resection prevented DSB mobility in G2. Similarly, in Drosophila, the activities of CtIP, Exo1, and Blm were necessary for DSB movement outside the heterochromatic domain (Chiolo et al., 2011). Conversely, we now show that inhibition of ARP2/3 decreases end-processing, consistent with positive feedback regulation between DSB mobility and resection. Indeed, DSB clustering could facilitate enzymatic reactions by increasing the local concentration of repair factors. Notably, in budding yeast, Arp2, Arp3, and Arpc2 have synthetic lethal genetic interactions with Mre11 and Sgs1 mutants (van Pel et al., 2013). This supports interdependency of actin polymerization and resection.

Our ChIP analyses suggest a two-step mechanism for actin nucleation at HDR sites (Fig. 2-20f). WASP is recruited at all DSBs regardless of cell cycle stage, reminiscent of NHEJ factors. By contrast, ARP2/3 is recruited in G2 to breaks that assemble HDR machinery. Actin polymerization promoted by WASP and ARP2/3 might therefore require sequential activation of PI(3)K-like kinases. Whereas ATM promotes the assembly of complexes required for resection, ATR
commits repair to HDR (Symington and Gautier, 2011). Accordingly, following ATM activation and WASP recruitment at DSBs, resection initiation could create a permissive environment for ARP2/3 activity leading to increased chromosomal mobility at specific DSB sites.

**METHODS**

**Chromatin binding assay and western blot**

Preparation of low-speed supernatant (LSS) Xenopus egg extracts and isolation of demembranated sperm nuclei (chromatin) were performed as previously described (Peterson et al., 2011). For chromatin binding experiments, 15 µl LSS extract was supplemented with demembranated sperm nuclei (2,500 sperm per µl) and incubated for 10 min at 21 °C before addition of DMSO or one of the following compounds for 10 min: CK-666 (160 µM), CK-548 (160 µM), KU5593 (100 µM), VE821 (50 µM). Subsequently, the PflMI restriction endonuclease was added to each sample (0.05 U/µl) before incubation for an additional 60 min at 21 °C. Extracts were diluted in chromatin isolation buffer (50 mM Hepes-KOH, pH 7.8, 100 mM KCl, 2.5 mM MgCl₂) supplemented with 0.125% Triton X-100 and overlaid on top of sucrose cushions (chromatin isolation buffer plus 30% sucrose) in 1.5-ml low-retention tubes (Thermo Fisher Scientific). Samples were spun at 7,180 r.p.m. for 30 min at 4 °C in a swing-bucket Sorval rotor (HB-6). Chromatin pellets were resuspended in 10 µl Laemmli buffer, boiled, and fractionated on a 4–22% Tris-glycine gel (Invitrogen) according to standard procedures, followed by transfer of resolved proteins onto PVDF membranes.
Following a 1-h block with 5% milk, membranes were incubated overnight at 4 °C with one of the following primary antibodies: β-actin (Sigma Aldrich: A5316, 1/1,000), ARPC4 (Novus Biologicals: NBP1-69003, 1/500), capping protein β-2 subunit (Developmental Studies Hybridoma Bank: E00007, 1/10,000), histone H3 (Cell Signaling Technology, 9715, 1/2,000) and Xenopus Mre1135. HRP-conjugated secondary antibodies were used (anti-rabbit IgG HRP, anti-mouse IgG HRP, Fisher Scientific) and chemiluminescence (Supersignal West Pico Chemiluminescent Substrate, 34077) was used.

**Mass spectrometric analysis of DSB-containing chromatin**

Chromatin samples were isolated as described above with the following modifications: 100 µl LSS extract was supplemented with sperm nuclei (5,000 sperm per µl) and incubated for 10 min at 21 °C before addition of PflMI restriction endonuclease (0.05 U/µl) and incubated for an additional 60 min. Chromatin was isolated through sucrose cushions as described above and extensively digested with 100 U micrococcal nuclease for 30 min at 37 °C (NEB). Chromatin proteins were reduced with DTT (5 mM, 30 min 60 °C) and alkylated with iodoacetamide (15 mM, 30 min, room temperature) before fractionation on SDS–PAGE. ‘In-gel’ digestion was performed with proteomic-grade trypsin (Promega) at 5 ng/µl for 16 h at 37 °C. The resulting digestion peptides were extracted from the gel pieces with 1:2 (vol:vol) 5% formic acid: acetonitrile solution. The resulting peptides were labelled with isobaric mass tags (ITRAQ 4-plex, Sciex), combined, purified with in-house made STAGE tips (Rappsilber et
resuspended in 0.5% acetic acid and loaded onto a home-packed reverse phase C18 column (75 µm ID). The peptides were separated using a linear gradient (0–42% acetonitrile, 0.5% acetic acid, 120 min, 150 nl/min) and directly sprayed into an LTQ-Orbitrap-Velos mass spectrometer for analysis (Thermo). The repetitive analytical cycle incorporated a high-resolution mass scan in the Orbitrap (resolution 30,000) followed by tandem MS scans of the five most intense peaks observed in each Orbitrap mass spectrum. Peptides were identified and quantified using Proteome Discoverer software (Thermo, Version 1.4).

**Cell culture and drug treatment**

U2OS and mouse-tail fibroblast cell lines were cultured in high-glucose Dulbecco’s modified Eagles medium supplemented with l-glutamine, 10% fetal bovine serum, and 1% penicillin-streptomycin. For neocarzinostatin-dependent DSB induction, cells were cultured on 8-well chamber slides (Thermo Fisher Scientific) and were treated with 0.5 µg/ml NCS for 60 min at 37 °C. Cells were washed twice with PBS before the addition of medium containing DMSO or one of the following compounds: CK-666 (Sigma Aldrich: SML-006, 100 µM), CK-548 (Sigma Aldrich: C7499, 50 µM), CK-689 (Sigma Aldrich: 18251750, 100 µM), and wiskostatin (Sigma Aldrich: W2270, 3 µM). After drug treatment, cells were then incubated at 37 °C for the indicated times. For mirin experiments, cells were pre-treated with mirin (Sigma Aldrich: M9948, 50 µM) for 1 h, before addition of NCS.
ER-AsiSI U2OS cells stably expressing the AsiSI restriction endonuclease tethered to the oestrogen receptor were provided by G. Legube (Centre de Biologie Integrative, Toulouse). For cell synchronization, cells were treated with 2 mM thymidine for two 18-h intervals separated by an 11-h release in fresh medium. Cells underwent double-thymidine block and were released into fresh medium for 7 h (G2) or 15 h (G1) before AsiSI-dependent DSB induction with medium supplemented with 300 nM 4-OHT (Sigma Aldrich, H7904) plus CK-666, CK-548, wiskostatin, or DMSO. Cells were subsequently incubated at 37 °C for 4 h.

ARPC2−/− fibroblast cell lines (provided by J. E. Bear, University of North Carolina) stably express the Cre-recombinase tagged to the oestrogen receptor. Upon 4-OHT treatment, the Arpc2 locus is floxed by Cre and cells are depleted of ARPC2 following 4 days of 4-OHT treatment.

CB33 and RD lymphoblastoid B cell lines were a gift from R. Dalla-Favera (Columbia University). Two B lymphocyte cell lines bearing distinct mutations in the WAS gene were obtained from the NIGMS Human Genetic Cell Repository (Coriell: GM1267, GM1268). GM1267 harbours a G>A transition at position 4 in intron 6 of the WAS gene (IVS6+5G>A). This mutation activates a cryptic splice site leading to splicing of a 38-nucleotide sequence from intron 6 onto exon 7. GM1268 harbours a G>A transition at nucleotide 257 in exon 2 on the WAS gene. This mutation results in a methionine for valine substitution at codon 75 (V75M). All B cell lines were grown in Rosewell Park Memorial Institute medium with 20% heat-inactivated bovine serum with 2 mM l-glutamine added.
Immunohistochemistry and quantification of DSB repair protein foci

U2OS cells were cultured on 8-well chamber slides (Thermo Fisher Scientific) and subjected to drug treatments as described above. Cells were then washed once with PBS before fixation with freshly prepared 4% PFA (pH 7.4) for 10 min. Cells were subsequently washed twice with PBS for 10 min before permeabilization with 0.1% PBS-Triton X-100 for 10 min. Cells were then washed once with PBS for 5 min before incubation with blocking buffer (3% BSA in 0.2% PBS-Tween) for 1 h. Cells were stained with primary antibodies diluted in blocking buffer under a Hybrislip (Invitrogen) overnight in a humidified chamber at 4 °C. Primary antibodies include γH2AX (EMD Millipore: 05-636, 1/500 or Abcam: ab81299, 1/500), WASP (Santa Cruz: sc-5300, 1/50), RAD51 (Santa Cruz: sc-8349, 1/50), 53BP1 (Novus Biologicals: NC100-304, 1/250) and FLAG (Sigma Aldrich: F3165, 1/250). Cells were then washed three times in PBS for 15 min and incubated with a fluorescence-conjugated secondary antibody in PBS with DAPI (Invitrogen, 1/10,000) for 1 h at room temperature. Secondary antibodies were Alexa 488 conjugated goat anti-mouse Ig (Abcam: ab150113, 1/1,000), Alexa 488 conjugated goat anti-rabbit Ig (Thermo Fisher Scientific, A-11034, 1/10,000), and Alexa 594 conjugated goat anti-mouse Ig (Thermo Fisher Scientific, A-11005, 1/10,000). Cells were then washed three times with PBS for 15 min. Vectashield was applied to each slide and slides were coverslipped. Slides were analysed under 40× magnification using a Zeiss Axio Imager Z2.
microscope, equipped with a CoolCube1 camera (Carl Zeiss). Images were processed for contrast enhancement and background reduction using ImageJ.

MetaCyte software (Metasystems, version 3.10.6) was used to detect U2OS nuclei based on DAPI staining and to perform automated quantification of \( \gamma \text{H2AX}, \text{RAD51}, \) and WASP foci within each nucleus. For analysis of the size and clustering of foci, images were converted to grey-scale and processed with Icy Software (Version 1.8.6.0; Institut Pasteur, Quantitative Image Analysis Unit; http://www.icy.bioimageanalysis.org). Regions of interest were drawn around individual nuclei and foci were detected using the Spot Detector plug-in (Channel 0) (Lagache et al., 2013). For clustering analyses, spots detected using Scale 2 (3 pixels were exported to the spatial analysis plug-in, which uses Ripley’s \( K \) function to assess the deviance of pairs of points from total randomness (Lagache et al., 2013; Caron et al., 2013). The software reports statistically significant clustering \( (P < 0.05) \) when the \( K \) function crosses the clustering threshold. The software further corrects for the number of points in the computations of the quantiles of the Ripley’s \( K \) function. Thus, there is no loss of statistical power for samples with lower numbers of foci. For foci size analysis, foci with minimum sizes of 1 and 3 pixels were counted using the Spot Detector plug-in. Scale 1 was used to detect foci that were at least 1 pixel in size, whereas Scale 2 was used to detect foci that were at least 3 pixels. Foci that met criteria for both Scale 1 and Scale 2 were excluded from the Scale 1 group to avoid duplicate detections. Foci counts were subsequently averaged to determine the mean size of foci per nucleus (100 nuclei were detected per condition or as
indicated). For co-localization studies, images were imported into Image J (NIH) and separated into green (γH2AX) and red (WASP) channels. These files were subsequently imported into Icy and regions of interest were drawn around individual nuclei. Pearson’s $r$ was calculated using the Co-localization Studio plug-in, which compares the coincidence of spots inside a region of interest between green and red channels.

**Chromatin immunoprecipitation**

ChIP experiments followed previously published protocols$^{20}$ with the following modifications: 50 million ER-AsiSI U2OS cells were cultured on 150-mm$^2$ plates in high-glucose Dulbecco’s modified Eagle’s medium supplemented with L-glutamine, 10% fetal bovine serum, and 1% penicillin-streptomycin. Following double-thymidine block, cells were released into fresh medium for 7 h (G2) or 15 h (G1) before incubation with 300 nM 4-OHT for 4 h. Cells were then trypsinized, washed with PBS, cross-linked in 1% methanol-free formaldehyde fixing buffer for 10 min, quenched with 0.125 M glycine for 5 min, and snap frozen at −80 °C. Cell lysis and nuclear isolation were performed using NP40 lysis buffer and SDS shearing buffer (Covaris). Nuclei were sonicated using the S220 Ultrasonicator (Covaris) to obtain chromatin fragments of 500–1,000 bp in length. Sheared chromatin was incubated with 10 µg antibodies to RAD51 (Santa Cruz: sc-8349), DNA-PKcs (Abcam: ab1832), WASP (Santa Cruz: sc-5300), ARPC2 (Santa Cruz: sc-32195) or IgG (Jackson ImmunoResearch Laboratories) overnight. These antibodies were validated for ChIP by previous studies (Taylor et al., 2010;
Aymard et al., 2014). Protein A/G magnetic beads were added overnight, followed by sequential washes at increasing stringency and reverse cross-linking at 65 °C. Immunoprecipitated DNA and input DNA were analysed in triplicate by qPCR. ChIP efficiencies (measured as per cent of input immunoprecipitated) were measured by qPCR at sites 80 bp downstream of DSBs. Graphs show ChIP efficiencies in G1, G2, or no 4-OHT cells, pooled from three experiments, and normalized against input material. Sequences for primers are listed below:

DSB I: 5’-GTCCCTCGAAGGGAGCAC-3’, 5’-CCGACTTTGCTGTGTGACC-3’;
DSB II: 5’-CCGCCAGAAAGTTTCCTAGA-3’, 5’-CTCACCCCTTGCAGCAGC-3’;
DSB III: 5’-TCCCCTGTTTTCATCTAGTT-3’, 5’-CTTCTGCTGTTCTGCGTCT-3’;
DSB IV: 5’-ATCGGGCCAATCTCAGAG-3’, 5’-GCGACGCTAAGTTAAAGCA-3’.

Primer pairs used to measure RAD51, DNA-PKcs, WASP, and ARPC2 were located 80 bp from DSB sites or at an undamaged genomic locus.

**I-Scel-induced DSB repair assays**

U2OS cell lines which harbour chromosomally integrated I-Scel-based GFP reporter substrates were used to monitor the efficiency of HDR, SSA, MMEJ, and c-NHEJ repair processes. The DR-GFP, SA-GFP, EJ2-GFP, and EJ5-GFP cell lines (gifts from J. Stark, City of Hope Cancer Center) were transfected with 1 µg I-Scel-expressing vector (FuGENE 6 transfection reagent (Promega), Opti-MEM (Gibco)) in medium supplemented with DMSO or one of the following compounds: CK-666 (Sigma Aldrich: SML-006, 50 µM), CK-548 (Sigma Aldrich:
C7499, 25 μM), CK-689 (Sigma Aldrich: 182517, 50 μM), SMIFH2 (Sigma Aldrich: S4826, 5 μM), or wiskostatin (Sigma Aldrich: W2270, 3 μM). The I-Scel-expressing vector was a gift from R. Baer. For the FLAG actin construct overexpression experiments, DR-GFP cells were transfected with FLAG–actin–NLS or FLAG–actin(R62D)–NLS for 48 h before transfection with I-Scel in medium supplemented with DMSO or CK-666. For the mCherry actin construct overexpression experiments, DR-GFP cells were co-transfected with I-Scel and mCherry–actin, mCherry–actin–NLS, mCherry–actin(R62D), or mCherry–actin(R62D)–NLS. Cells were harvested 48 h after I-Scel transfection and percentage GFP+ events were counted by fluorescence-activated cell sorting (FACS) (10,000 cells per biological replicate). For all conditions, parallel transfection with 1 μg pEGFP-N3 vector (Clontech) was used to determine transfection efficiency. Percentage refers to the number of GFP+ cells divided by the number of pEGFP+ cells.

Resection assay
A detailed protocol for the resection assay has been recently described (Zhou et al., 2014). In brief, ER-AsiSI U2OS cells were treated with 300 nM 4-OHT in medium supplemented with DMSO, CK-548 (50 μM), or CK-666 (100 μM). For mirin experiments, cells were pre-treated with mirin (Sigma Aldrich: M9948, 50 μM) for 1 h before addition of 4-OHT, and subsequently washed and incubated with mirin for the indicated times. After 4 h, cells were trypsinized and washed with cold PBS before resuspension in lysis buffer (100 mM NaCl, 10 mM TrisCl
pH 8, 25 mM EDTA, pH 8, 0.5% SDS, 0.1 mg/ml proteinase K). Cells were agitated at 37 °C overnight. After phenol/chloroform purification and salt/ethanol extraction, precipitated DNA underwent restriction enzyme digestion by BamHI-HF, BsrGI, or HindIII-HF (1 unit enzyme per 7 ng DNA) at 37 °C overnight. DNA digests were subsequently used as templates in a 20 µl qPCR reaction containing 10.0 µl 2× TaqMan Universal PCR Master mix (Thermo), 0.5 µM of each primer, and 0.2 µM of probe. The extent of ssDNA generation at sites downstream of various DSBs was determined by calculating the ∆Ct value for each site (mock-digested Ct value − digest Ct value) and using the following equation: ssDNA = 1/(2^{(∆Ct − 1) + 0.5}) × 100.

Sequences for primers and probes are listed below: DSB V (335 bp site): 5'-GAATCGGATGTATGCGACTGATC-3', 5'-TTCCAAAGTTATTTCCAACCCGAT-3'; 6FAM-CACAGCTTGCCCATCCTTGCAAACC-TAMRA; DSB V (1618 bp site): 5'-TGAGGAGGTGACATTAGAACTCAGA-3', 5'-AGGACTCACTTACACGGCCTTT-3'; 6FAM-TTGCAAGGCTGCTTCTTACCATTCAA-TAMRA; DSB V (3500 bp site): 5'- TCCTAGCCGATAATATAGCTATACAAACA-3', 5'-TGAATAGACAGACAAACAGATAATGAGACA-3'; 6FAM-ACCCTGATCGCCTTTCCATGGGAATAG-TAMRA; DSB VI (364 bp site): 5'-CCAGCAGTAAGGAGGACAGA-3', 5'-CTGTCAATCGTCTGCCCTTC-3'; 6FAM-CCAGGCCCTCAAAATCCTCCTCAGT-TAMRA; DSB VI (1754 bp site): 5'-GAAGCCATCCTACTCTTCTTCACCT-3', 5'-GCTGGAGATGATGAAGCC-3'; 6FAM-CACTCCCTGTTTCTTCTTGCTCCGA-TAMRA; DSB VI (3564 bp site):
site): 5’-GCCCAAGCTAAGATCTTCCTTCA-3’, 5’-

CTCTTTGCCCCTGAGAAGTGA-3’; 6FAM-CTGCAGCCCTCAAGCCCGGAT-

TAMRA. No DSB site: 5’- ATTGGGTATCTGCGTCTAGTGAGG-3’, 5’-

GACTCAATTACATCCCTGCAGCT-3’; 6FAM-

TCTCTGCACAGACCGGCTTCCCTC-TAMRA.

**AsiSI-AID repair assay**

The repair assay followed recently published protocols with the following modifications (Aymard et al., 2014; Zhou et al., 2014). Five million ER-AsiSI-AID U2OS cells were cultured on 100-mm² plates in high-glucose Dulbecco’s modified Eagle’s medium supplemented with l-glutamine, 10% fetal bovine serum, and 1% penicillin-streptomycin. Following double-thymidine block, cells were released into fresh medium for 7 h (G2) or 15 h (G1) before incubation with 300 nM 4-OHT and DMSO or CK-666 (100 µM) for 4 h. Cells were subsequently washed three times with PBS and then incubated in medium containing auxin (Sigma: I5148, 500 µg/ml) and DMSO or CK-666. Cells were harvested 0, 2, or 8 h after auxin exposure. Phenol/chloroform purification followed by salt/ethanol extraction was performed to precipitate DNA. DNA were subsequently used as templates in a 20-µl qPCR reaction containing 10.0 µl 2× TaqMan Universal PCR Master mix (Thermo), 0.5 µM of each primer, and 0.2 µM of probe. The level of unrepaired DSBs was determined by normalizing the Ct value for each DSB site with a control, undamaged site. Sequences for primers and probes are listed.
below. DSB V (across DSB): 5′-GATGTGGCCAGGGATTGG-3′, 5′-
CACTCAAGCCCAACCCGT-3′; DSB VI (across DSB): 5′-
GAGGAGCCTCTCCTGCAGC-3′, 5′-GAACCAGACCTACCTCCAGGG-3′.

Live cell imaging

U2OS cells stably expressing RAD52–mCherry, 53BP1–YFP, and Geminin–CFP constructs (Karanam et al., 2012) were cultured on 35-mm glass bottom microwell dishes (MatTek, P35GC-1.5-10-C). Cells were treated with 0.5 µg/ml NCS to induce DSBs for 60 min at 37 °C. Subsequently, cells were washed twice with PBS before the addition of medium containing DMSO or CK-666 (100 µM). For mirin experiments, cells were pre-treated with mirin (Sigma Aldrich: M9948, 50 µM) for 1 h before addition of NCS, and subsequently washed and incubated with mirin for the indicated times. S-phase cells were selected for RAD52 foci analysis by screening for Geminin–CFP positivity. G1 cells were selected for 53BP1 foci analysis by screening for Geminin–CFP+/RAD52–mCherry+ cells. After 20 h, images were acquired on an A1RMP confocal microscope (Nikon Instruments), on a TiE Eclipse stand equipped with a 60×/1.49 Apo-TIRF oil-immersion objective lens, an automated XY stage, stage-mounted piezoelectric focus drive, and a heated, humidified stagetop chamber with 5% CO₂ atmosphere. Cells expressing RAD52–mCherry and 53BP1–YFP foci were imaged in GaAsP detectors using 561 nm and 488 nm excitation, respectively, and standard RFP and GFP emission filters. The confocal pinhole was set to 1 Airy unit for the red channel. Foci movements were examined by collecting z
series at 0.4-μm intervals throughout the entire nucleus every 5 min for 2.5 h. Focus was maintained by the Perfect Focus System (Nikon).

**Nuclear actin-chromobody TagGFP imaging and analysis**

For live cell imaging experiments, U2OS cells were cultured on 35-mm glass bottom microwell dishes at 90% confluency (MatTek, P35GC-1.5-10-C) and transfected with 0.3 μg of vector expressing nuclear actin-cb (Chromotek, acg-n; EMD Millipore, FuGENE 6 transfection reagent; Promega) After 6 h, cells were treated with 1 μg/ml NCS for 60 min at 37 °C. Cells were washed twice with PBS before the addition of medium containing DMSO. After 3 h, images were acquired on an A1RMP confocal microscope using the equipment and settings described above. Cells with actin-cb foci were classified using the following criteria: actin-cb foci exhibited movement and frequent turnover, were round in morphology and often appeared to have fuzzy ‘tails’. Cells with actin-cb rods were classified using the following criteria: actin-cb rods exhibited limited movement, no turnover, were very bright, and were filamentous in morphology. Foci movements were examined by collecting z series at 0.4-μm intervals throughout the entire nucleus every 30 s for 10 min. Following initial image acquisition, cells were subsequently treated with 100 μM CK-666. Images of actin-cb foci were subsequently acquired 1 h post CK-666 treatment. Actin-cb foci were scored as the number of discrete punctate structures appearing per nucleus, as detected by the Icy Spot Detector plug-in. For experiments assessing localization of actin-cb foci with RPA, U2OS cells were co-transfected with actin-
cb and RPA32–NLS–mCherry (a gift from J. Lukas) for 16 h before NCS treatment. After 12 h, foci movements were examined by collecting z series at 0.4-μm intervals throughout the entire nucleus every 5 min for 2.5 h.

For experiments in fixed cells, U2OS cells were cultured on 8-well chamber slides (Thermo Fisher Scientific), subjected to transfection and drug treatments as described above, and processed for IHC as described above. Cells were stained with primary antibodies against RAD51 (Santa Cruz: sc-8349, 1/50) and GFP (Abcam: ab13970, 1/100) overnight. Secondary antibodies were Alexa 488 conjugated goat anti-chicken Ig (Invitrogen: A11039, 1/1000) and Alexa 594 conjugated goat anti-mouse Ig (Thermo Fisher Scientific, A-11005, 1/10000). Actin-cb-expressing cells were selected for analysis by screening for actin-cb foci. Images were acquired on an A1RMP confocal microscope equipped with a 60×/1.49 Apo-TIRF oil-immersion objective lens. Z series at 0.4-μm intervals throughout the entire nucleus were acquired. For co-localization analysis, actin-cb and Rad51 foci were detected in z using the spot detector plug-in available on Icy Bioimaging Software platform. The co-localization of RAD51 and actin-cb spots was assessed using the Co-localization Studio plug-in, object-based methodology. Here, co-localization was defined as the number of RAD51 spots that directly overlapped with or touched actin-cb foci (within a 0.7-μm radius).

**Analysis of movement of DSB foci**
DSB movement analysis followed the approach recently described (Cho et al., 2014). Nikon NIS Elements data files were processed in ImageJ (Schindelin et al., 2012). Green and red channels were split and a maximum-intensity projection of each z stack was generated. T stacks were subsequently aligned using the StackReg plugin (Thevenaz et al., 1998) to correct for cell movements over the duration of the experiment. Cells that underwent large-scale nuclear deformations or expansions were discarded. The TrackMate plug-in for ImageJ (Tinevez et al., 2017) was used to perform single-particle tracking of DSB foci over a 100-min interval and monitor foci intensity. Focus trajectories were subsequently exported to Matlab and analysed using the class @msdanalyzer (Tarantino et al., 2014). The MSD of DNA damage foci plots the average squared distance travelled by foci at increasing time intervals, whereas the diffusion coefficient \( D(t) \) is approximated through the linear-weighted fit of the initial mean MSD curve\(^4\). MSD curves for DSB foci in a given cell were computed using the formula \( \text{MSD} = (x(t + \Delta t) - x(t))^2 \), where \( x \) reflects focus position and \( t \) is the time in minutes. For RAD52 foci, the weighted mean of all the MSD curves acquired from >2,000 tracks in three independent experiments is shown. The error bars for each point on the weighted mean of the MSD curve represent the weighted s.e.m. over all MSD curves. The diffusion coefficient \( D(t) \) was estimated from the linear fit of the first 20% of each MSD curve. Calculation of the time-dependence coefficient \( \alpha \) was performed using Matlab via log–log fitting of the power law \( \text{MSD}(t) = \Gamma \times t^\alpha \). Cumulative distance (CD) was calculated by summing the distances foci travelled over 100 min relative to their starting position using the
formula: \[ D(i) = \sqrt{(X(i) - X(i-1))^2 + (Y(i) - Y(i-1))^2} \] where \( X \) and \( Y \) refer to the \( x \) and \( y \) coordinates of the focus at time \( i \).

**Analysis of clustering of DSB foci**

For analysis of clustering of DSB foci during live cell imaging, a clustering event was defined as complete co-localization of \( \geq 2 \) foci over \( \geq 3 \) consecutive frames (15 min) over a 100-min interval. For actin-cb clustering during live cell imaging, a clustering event was defined as complete co-localization of \( \geq 2 \) foci over \( \geq 3 \) consecutive frames (3 min) over a 20-min interval.

**Quantification of RPA association to chromatin by flow cytometry**

Detection of chromatin-bound RPA in S-phase cells followed recently published protocols with the following modifications: \( 2 \times 10^6 \) cells were treated with DMSO or \( 1 \) \( \mu \)g/ml camptothecin (CPT) for 24 h. Cells were subsequently washed once with cold PBS, then incubated in cold CSK buffer + 1% Triton-X for 5 min. Cells were then washed with PBS and fixed in 0.5% PFA for 15 min. Cells were subsequently washed twice in BD Perm/Wash buffer (Becton Dickinson: BD554723) and stained with RPA2/RPA32 antibody (Abcam: ab2175, 1/50) overnight. Cells were then washed with PBS and incubated in secondary antibody (Abcam: ab150113, 1/50 or Alexa 647 conjugated donkey anti-mouse Ig, Invitrogen: A31571, 1/50) for 1 h. Cells were then washed and stained with propidium iodide (PI) in the presence of RNase A overnight. The percentage of S-phase cells as identified by PI staining was quantified by flow cytometry. The
gate for RPA-positive cells was established using a negative control sample that was stained with mouse IgG following extraction. RPA-positive cells following DMSO and CPT treatment were subsequently quantified by flow cytometry. A minimum of 10,000 cells was counted per biological replicate.

Clonogenic and cell survival assays

U2OS cells were seeded onto 10-cm dishes (1,000 cells per dish) overnight. Cells were subsequently treated with 0, 100, 200, or 300 µM CK-666 in the presence of DMSO, camptothecin (CPT, 10 nM), or aphidocolin (APH, 400 nM) for 12 h. Cells were then washed with PBS and incubated in fresh medium for 10–14 days. Cells were subsequently washed with PBS, fixed for 10 min with 100% methanol, and stained with 0.1% crystal violet for 10 min. For olaparib studies, cells were treated with 0, 10, or 20 µM CK-666 in the presence of DMSO or 50 nM olaparib for 14 days, and fixed and stained as above. Each experiment was performed in triplicate.

For colony quantification, images of dishes were converted to grey-scale and processed with Icy Software (Version 1.8.6.0; Institut Pasteur, Quantitative Image Analysis Unit; http://www.icy.bioimageanalysis.org). Regions of interest were drawn around individual dishes and colonies were counted using the Spot Detector plug-in (Channel 0). Counts from two biological replicates were normalized for plating efficiency.
For the annexin V/PI survival assay, 1–5 × 10^5 cells were incubated in DMSO or 1 μg/ml CPT for 0, 12, or 24 h. Cells were then washed in 1× binding buffer (Thermo Fisher Scientific: 00-0055) and stained with APC-conjugated annexin V (Thermo Fisher Scientific: 17-8007) for 10 min. Cells were then washed in 1× binding buffer and stained in PI (Thermo Fisher Scientific: 00-6990). Per cent viability following CPT treatment was assessed by measuring the percentage of APC-Annexin V-negative and PI-negative cells by flow cytometry. A minimum of 10,000 cells counted per condition.

Quantification and statistical analysis
Statistical parameters are reported in the Figures and legends. Two-tailed t-tests and ordinary one-way ANOVAs were used for comparisons of means of normally distributed data and two-tailed Mann–Whitney U tests were used for comparison of non-normally distributed data. Statistical analyses were performed using Prism 7.0. No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.
Chapter 3: Conclusion
DISCUSSION AND FUTURE DIRECTIONS:

Over the past fifteen years, DNA double-strand break (DSB) mobility has been demonstrated in budding yeast, Drosophila, and mammalian cells. Many of the proteins that carry out DSB repair also regulate break movement. For example, Rad51 loading at breaks destined for HDR stiffens the chromatin fiber (Miné-Hattab et al., 2017). This permits the break to efficiently navigate the chromatin meshwork in the search for a homologous partner. In contrast, DNA decompaction by chromatin remodeling complexes frees the break from nucleosomal constraints (Neumann et al., 2012; Seeber et al., 2013). This relaxation of damaged and undamaged chromosomes increases genome fluidity. However, whether active forces set repair domains in motion has remained an open question. Our results are consistent with a model in which WASP activates Arp2/3 to polymerize actin at resected DSBs destined for HDR (Fig. 3-1). Activation of Arp2/3 by WASP specifically induces movement at these damaged loci. This promotes DSB clustering which in turn facilitates DNA end-processing and repair.
Fig. 3-1 | Arp2/3 polymerizes actin during HDR to drive foci clustering.
**Fig. 3-1 | Arp2/3 polymerizes actin during HDR to drive foci clustering.** A mammalian nucleus is shown with red and yellow dots reflecting sites of HDR and NHEj, respectively. Green blobs represent sites of DNA damage-induced nuclear actin polymerization. Dendritic arbors nucleated by WASP and Arp2/3 push chromatin thereby creating a path for damaged sites to find one another and coalesce (upper box). Notably, DSBs that repair by NHEJ do not require clustering by Arp2/3 and therefore do not nucleate actin (lower box).
A role for DSB clustering in DNA end-resection

In yeast, DSBs roam a nuclear volume that is ten-fold more expansive than the volume occupied by undamaged chromosomes (Lisby et al., 2003; Dion et al., 2012; Miné-Hattab and Rothstein, 2012). It has been proposed that DSB mobility facilitates the search for a homologous chromosome during HDR (Smith and Rothstein, 2017). This concept is supported by studies in ALT cells in which Rad51-coated telomeres undergo directed movements over long distances to find templates for repair (Cho et al., 2014). In contrast to recombination between homologs, chromosomal DSBs undergoing HDR frequently utilize the sister chromatid as a repair template (Johnson and Jasin, 2000). Given that sister chromatids are tightly bound by cohesin, a genome-wide search for a homologous template is not warranted, and indeed mammalian DSBs explore a fraction of the nuclear volume relative to yeast (Krawczyk et al., 2012). Instead, DSBs in mammalian cells show a tendency to merge into repair clusters (Aten et al., 2004; Caron et al., 2015; Aymard et al., 2017). Unlike homology search which pairs damaged and undamaged loci, DSB clustering occurs when multiple migrating break sites coalesce. This movement preferentially occurs at DSBs undergoing HDR (Aymard et al., 2017; Schrank et al., 2018). How DSB clustering might serve a requisite step in repair, however, has remained a matter of speculation.

DSB resection must be restrained to prevent mutagenic single-strand annealing between complementary strands (Ochs et al., 2016). On the other hand, sufficient end processing is required to reveal sequences for homolog
recognition. The degree to which a DSB is resected may depend on extent of DNA damage. DSBs that utilize an undamaged sister chromatid for repair undergo HDR without long-range resection (Westmoreland and Resnick, 2016). However, excess DNA damage may destroy target templates and necessitate more resection to reveal distinct sequences for homology search. DSB clustering is one mechanism by which end processing might be tuned to the degree of damage incurred by the genome. While mammalian cells with a handful of breaks exhibit minimal chromosomal mobility (Roukos et al., 2013), cells that suffer hundreds of DSBs have enhanced motion and clustering (Aten et al., 2004; Neumaier et al., 2012; Caron et al., 2015; Aymard et al., 2017). Similarly, DSB movement in yeast positively correlates with more extensive DNA damage (Mine Hattab and Rothstein, 2012). Increased chromosomal motion could promote clustering of damaged chromosomes leading in turn to more efficient end processing by concentrated repair machinery (Fig. 3-2). Indeed, in Chapter 2, we show that cells that express the AsiSI restriction enzyme (which generates ~100 γH2AX foci) or cells treated with NCS (which generates ~50 γH2AX foci) exhibit clustering of resected breaks. Whether DNA damage and DSB movement are positively correlated to facilitate clustering may be tested by comparing the movement of NHEJ breaks in G1 cells versus HDR breaks in G2: Greater DSB mobility and clustering with increasing DNA damage in G2 but not G1 cells would support the notion that DSB clustering is tuned to the level of DNA damage and is a specific feature of HDR.
Enhanced motion by Arp2/3 specifically targets HDR breaks suggesting that DSBs requiring minimal resection like NHEJ or MMEJ need not cluster. In line with this notion, we show that Arp2/3 inactivation does not block the repair kinetics of AsiSI-induced DSBs in G1 cells which utilize NHEJ for repair. However, Arp2/3 activity is required for the long-range resection and repair of DSBs in G2 AsiSI cells. Relatedly, etoposide-induced breaks that require end processing for resolution move faster than simpler DSB lesions (Krawczyk et al., 2012). Thus, DSB clustering may reflect an effort to resolve bulky adducts that require resection.

Finally, increased global mobility upon DSB generation requires the formation of Rad51 nucleoprotein filaments (Smith et al., 2018). Since Rad51 binds resected breaks and Arp2/3 facilitates end resection, it is possible that the defect in DSB mobility observed upon Arp2/3 inhibition reflects impaired stiffening of the chromatin fiber which accompanies Rad51 loading. Thus, the effect of Arp2/3 inhibition on RPA dynamics should be tested in the setting of Rad51 knockdown. However, several lines of evidence point towards a role for actin polymerization machinery in DSB movement that is uncoupled from Rad51 activity. For example, DSBs destined for HDR cluster in G1 prior to binding Rad51 in an actin-dependent manner (Aymard et al., 2017). Additionally, Arp2/3 promotes the migration of resected DSBs from the heterochromatic compartment to the nuclear periphery where Rad51 loading occurs (Caridi et al., 2018). Thus, DSB resection may create a permissive environment for Arp2/3 activation promoting clustering and ultimately Rad51 enrichment on chromatin.
Fig. 3-2 | Clustering facilitates extensive DSB end-resection.
Fig. 3-2 | Clustering facilitates extensive DSB end-resection. Clustering of non-homologous chromosomes is shown with red tracks denoting movements and red and green dots reflecting damaged and undamaged sites, respectively. This pathway is active in S and G2 phase. Mitotic chromosomes are shown to distinguish sister chromatids from chromosomal homologs. Clustering between damaged ends may concentrate repair machinery in centers thereby increasing the efficiency of requisite HDR steps (red lines shown represent paths of clustering foci). In contrast, DSBs undergoing NHEJ have limited motion and do not cluster. Clustering of ends could favor enhanced resection to expose longer tracts for homology search. This might prove especially important when the genome suffers extensive damage which disrupts templates on potential sister chromatid and interhomolog sites. Following resection, homology search between sister chromatids is shown (red-to-green lines indicate foci paths). If end resection exposes sufficient sequences for homology, strand invasion and HDR heals the broken lesion.
A role for DSB clustering in the genesis of translocations

The genes encoding the Arp2/3 complex are frequently amplified in colorectal, breast, ovarian, bladder and esophageal tumors analyzed in large patient cohorts (Molinie and Gautreau, 2018; cbioportal.org). Moreover, the Cancer Genome Atlas has identified mutant WASP as one of 299 genes that drive cancer across 9,423 tumors from over thirty different cancer types (Bailey et al., 2018). Deregulation of the Arp2/3 regulatory system has been proposed to promote tumor cell invadopodia leading to metastasis (Yamaguchi and Condeelis, 2007). Alternatively, Arp2/3-driven DSB movement and clustering might affect chromosomal translocations that drive oncogenesis (Fig. 3-3). Indeed, direct observation of translocating DSBs reveal they are highly mobile relative to breaks undergoing NHEJ (Roukos et al., 2013). Moreover, indirect evidence suggests that dynamic chromosomal loci (e.g. those that migrate to transcription factories) are recurrent substrates for translocation (Klein et al., 2011). Given that WASP, Arp2/3, and F-actin localize to active gene promoters (Taylor et al., 2010) and DSBs that cluster for HDR (Chapter 2), the impact of nuclear actin dynamics on translocation is ripe for investigation. These experiments may be carried out by high-throughput genome-wide translocation sequencing (HTGTS) of site-specific DSBs in the AsiSI cell line (Aymard et al., 2014; Hu et al., 2016). Here, translocations between a bait site and genome-wide “prey” DSBs induced by AsiSI may be monitored in G2 when Arp2/3 is active or in conditions when Arp2/3 is inactive (e.g. in G1 or in CK-666 treated cells).
The incidence of chromosomal translocations should also be evaluated in the setting of constitutively active WASP. In Chapter 2, we show that WASP is recruited at all DSBs regardless of cell cycle stage. In contrast, Arp2/3 is recruited in G2 to a subset of breaks that assemble HDR machinery, e.g. RPA, Rad51 and Rad52. This two-step mechanism for regulating actin polymerization has precedence at the leading edge: the WASP-interacting protein (WIP) localizes inactive WASP to potential sites of Arp2/3 nucleation (Martinez-Quiles et al., 2001; Ho et al., 2004; Chou et al., 2006). Mutations in the GTPase binding domain of WASP (L270P, S272P, and I294T) disrupts its autoinhibitory interaction with the VCA domain leading to unscheduled activation of Arp2/3 (Devriendt et al., 2001). It would be therefore interesting to test whether hyperactive WASP enhances DSB mobility particularly at NHEJ breaks where it is presumed to be inactive. Indeed, WASP I294T B and T cells exhibit elevated DSBs, doublet and fused chromosomes, and tetraploidy indicating a heightened state of genome instability (Westerberg et al., 2010). Accordingly, HTGTS should be performed in cells that overexpress hyperactive WASP to assess whether actin-driven movements promote translocations.

Alternatively, it is also conceivable DSB clustering shields breaks from translocation. Moreover, a failure to cluster DSBs could lead to inefficient DSB end resection favoring MMEJ of short ssDNA overhangs. In either case, Arp2/3 inhibition would increase the frequency of translocating DSBs.
Fig. 3-3 | DSB clustering may promote or prevent chromosomal translocations.
Fig. 3-3 | DSB clustering may promote or prevent chromosomal translocations. a) Chromosomal translocations that form oncogenic gene chimeras or disrupt the function of tumor suppressors promote tumorigenesis. Clustering between DSBs undergoing resection could facilitate the illegitimate joining of non-homologous chromosomal ends (right arrow). Enhanced resection could expose regions of homology that could be ligated by MMEJ/SSA machinery throughout interphase. Alternatively, clustering may shield DSBs undergoing HDR from breaks undergoing NHEJ or MMEJ (left arrow). In this setting, DSB clustering would prevent mutagenic rearrangements.
Targeting Nuclear WASP and Arp2/3 For Cancer Therapy

Unlike activating mutations, loss of function mutations in the WAS family of proteins are associated with a number of cancerous malignancies, particularly lymphomas and leukemias. Anecdotal evidence suggests that WAS carriers are more susceptible to breast and thyroid cancers (Sumathi Iyengar, personal communications). Accordingly, by mediating HDR via DSB clustering, WASP may serve an essential tumor suppressor role. Thus, loss of WASP activity could drive oncogenesis. Along the same lines, overexpression of Arp2/3 subunits in tumor cells might produce defective or incomplete complexes. Indeed, amplification of Arp2/3 genes in tumors generally affects only one subunit of the complex (cbiportal.org). Arp2/3 subunit overexpression does not necessarily yield multiple complexes given the stoichiometry of the six other subunits. Moreover, overexpression of a single subunit might cause a dominant-negative effect. For example, Arpc1/Arpc5 form a stable heterodimer in the absence of other subunits (Gournier et al., 2001). Accordingly, overexpression of Arpc1 could sequester Arpc5 from incorporation into Arp2/3 complexes. This could consequently decrease DSB clustering by Arp2/3 as Arp2/3 complexes that lack Arpc5/Arpc1 subunits have decreased efficiency of actin nucleation. Thus, loss of Arp2/3 or WASP activity may promote oncogenesis by decreasing the efficiency of HDR.

On the other hand, tumor cells with ongoing levels of genomic instability and DNA replication stress might be specifically sensitive to Arp2/3 inactivation. Indeed, in Chapter 2, we show that CK-666 sensitizes cells to replication stress induced by aphidicolin and DSBs generated in S-phase by etoposide. Tumors
can tolerate otherwise lethal levels of DNA damage by exploiting DNA repair pathways. Thus, discovering new strategies to selectively inhibit the repair of DSBs remains a major goal in the development of more effective cancer therapies. These preliminary studies suggest that CK compounds are promising radio and chemo-sensitizing agents to be tested in conjunction with other DNA damaging therapies.

*Dissecting WASP-Arp2/3 regulation at DSBs*

In mammalian cells DSB movements require CtIP (Aymard et al., 2017) and exonucleolytic processing by Mre11 (our study), while in yeast Sae2 deletion delays chromatin motion (Mine-Hattab and Rothstein, 2012). Taken together, these data suggest that resection enhances chromosome mobility. However, we have conversely shown that inhibiting movement by blocking Arp2/3 decreases end resection. These data suggest a positive feedback loop in which resection enhances Arp2/3-driven DSB movements, which, in turn, enhance resection. This interdependence between actin polymerization and end processing makes the study of Arp2/3 activation by DNA damage signaling quite complex. For example, many drug treatments that impact Arp2/3 enrichment on chromatin also restrict resection. In Chapter 2, we show that Arp2/3 recruitment requires the PIKKs ATM and ATR in *Xenopus* extracts. However, we concomitantly observe a decrease in RPA enrichment on chromatin when ATM and ATR are inactivated. Thus, it is unclear whether ATM and ATR directly regulate WASP-Arp2/3 activation or simply reduce the ssDNA generated by resection machinery.
Actin polymerization at DSBs likely occurs by a two-step mechanism in which inactive WASP localizes to DSB sites and activates Arp2/3 during HDR. Monitoring the localization of WASP and Arp2/3 by ChIP may yield further insight into their regulation. For example, ATM depletion may abolish both WASP and Arp2/3 enrichment at DSB sites, whereas loss of ATR signaling may selectively block Arp2/3 recruitment and activation. Similarly, ChIP may also be used to interrogate the localization of other WASP-Arp2/3 regulators, including WIP, cofilin, and capping protein. Finally, the influence of PIKKs and other cell cycle regulators on DSB mobility may also be studied by live cell imaging of damaged cells expressing the nuclear actin chromobody. As will be discussed below, DSBs assemble dynamic actin clusters which reflect functional Arp2/3 activity.

*Nuclear actin polymerization: Relating structure to function*

Early studies of nuclear actin polymerization identified a variety of actin structures that arose following DNA damage in cells expressing LifeAct (Belin et al., 2015). Elongated filaments were shown to require the formin family of nucleators whereas amorphous “clusters” were polymerized by unknown mechanisms. In our study elongated actin rods and actin foci (i.e. clusters) were also identified in the nucleoplasm of damaged cells expressing the chromobody construct. However, live cell imaging of elongated rods showed minimal movement while actin foci were highly dynamic. Moreover, CK-666 abolished nuclear actin foci, whereas nuclear actin rods continued to grow. These data suggest that formins and Arp2/3 may compete for actin monomers following DSB
generation to form structures with distinct purposes. Indeed, while actin foci overlap with sites of DNA damage, we and others (Belin et al., 2015) observe minimal colocalization of nuclear actin filaments with repair foci. Formin-induced filaments may regulate the redox state of the nucleus following DNA damage or facilitate the transcription of DSB-induced genes akin to its role in regulating SRF activity (Baarlink et al., 2013).

In contrast Arp2/3 generates propulsive forces by nucleating branched actin structures. These actin networks may push DSBs directly or jostle neighboring domains thereby allowing breaks to migrate the chromatin meshwork more freely (Fig. 3-1). Notably, tethering chromatin remodeling complexes to DNA relaxes the chromatin fiber such that chromosomes display nearly free diffusion (Neumann et al., 2012). In contrast, we show that HDR breaks move faster than NHEJ breaks albeit with confined Brownian motion. This indicates that nuclear actin polymerization doesn’t dramatically alter the physical properties of chromatin but might transduce movement by colliding around the break site. In contrast, DSBs that arise in repressive regions such as Drosophila heterochromatin or the nucleolus exhibit long-directed movements. These DSBs also harness Arp2/3 to polymerize nuclear actin complexes but require myosins I and V to transit breaks to the nuclear periphery (Caridi et al., 2018). Thus, directed motion may require processive transport by myosin along actin structures. Future studies that employ super-resolution microscopy will be required to determine how nuclear actin filaments are tethered to transiting chromatin.
Concluding Remarks

Many studies have found that DSBs induce chromatin motion. The molecular basis for DSB movement and the role of clustering in DNA damage repair, however, were enigmatic. My studies using *Xenopus* cell free extracts and mammalian cells have shown how DSBs harness nuclear actin polymerization machinery to drive DSB motion. Notably, inhibition of actin polymerization by inactivation of WASP or Arp2/3 reduces the clustering and repair of DSBs undergoing HDR. Taken together, these studies contribute to our knowledge of how cells repair DSBs throughout the cell cycle. Future endeavors to understand how these mechanisms are regulated during transcription and replication, when HDR is obligatory, will help develop a deeper appreciation of the role nuclear actin polymerization might play beyond repair.
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