Multi-level dynamics of heterochromatin and repair sites undergoing homologous recombination

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Abstract

Studies across different organisms show that nuclear architecture and dynamics play central roles in different aspects of homologous recombination (HR) repair. Here we review the most recent discoveries in this field, ranging from directed motions mediating relocalization pathways, to global chromatin mobilization, local DNA looping, and changes in repair focus properties associated with clustering and phase separation. We will highlight how these dynamics work in different contexts, including the molecular mechanisms and regulatory pathways involved. We will also discuss how they function in pericentromeric heterochromatin, which presents a unique environment for HR repair given the abundance of repeated DNA sequences prone to aberrant recombination, the 'silent' chromatin state, and the phase separation characterizing this domain.

Highlights

- Nuclear actin filaments and myosins drive the directed motions of heterochromatic repair sites
- Local and global changes in heterochromatin dynamics promote DNA damage signaling and repair
- Pre-existing topological organization of the chromatin, and damage-induced changes in this organization, contribute to local dynamics and DSB repair
- Clustering of DSBs promote early repair steps
- Phase separation influences several aspects of DSB repair, including dynamics

Short title

New insights into heterochromatin repair dynamics

Keywords

Homologous recombination, Heterochromatin, Phase separation, Nuclear dynamics, Nuclear architecture

Introduction

HR is a primary double-strand break (DSB) repair pathway, in which homologous sequences on the sister chromatid or the homologous chromosome provide templates for DNA synthesis and repair. A first level of nuclear dynamics during HR relates to the 'homology search' step, where the Rad51-coated nucleofilament finds its template in the genome. When the sister chromatid is used, it is typically in close proximity due to cohesion, resulting in a local search and minimal dynamics. However, when the homologous chromosome is used, the nucleofilament might need to travel for a long distance (reviewed in^[1]). Additionally, 'safe' HR of pericentromeric heterochromatin (hereafter 'heterochromatin') and other repeated sequences at high risk for aberrant recombination, requires the isolation of repair sites to new nuclear locations, resulting in extensive dynamics (reviewed in^[2,3]). This review focuses on emerging concepts in the field of nuclear dynamics of repair foci, including: i) directed motions of certain repair sites to distant locations; ii) local and global changes in chromatin compaction contributing to dynamics; and iii) local changes in dynamics related to DNA looping, focus clustering, and phase separation. Notably, components required for these dynamics are frequently misregulated in cancer cells, cancer-prone genetic disorders, neurodegenerative diseases, neuromuscular disorders, and deteriorate with age (reviewed in^[2-4]). Thus, understanding the molecular mechanisms driving these dynamics is expected to have a major impact on the future development of therapeutic strategies for a variety of human disorders.

Relocalization of heterochromatic DSBs and other repeated sequences

Heterochromatin presents unique challenges to HR repair, and is an interesting case study for understanding nuclear dynamics. Heterochromatin occupies about 30% of fly and human genomes, and is mostly composed of repeated sequences prone to ectopic recombination (reviewed in^[3]). In *Drosophila*, about half of these sequences are 'satellite' repeats (predominantly 5-base pair sequences repeated for hundreds of kilobases to megabases) and the rest are transposable elements and other scrambled repeats (reviewed in^[3,5]). Importantly, heterochromatin is functionally and structurally distinct from lamina-associated domains (LADs) and is typically not associated with the nuclear periphery^[6-11]. Studies in *Drosophila* cells, where heterochromatin forms a distinct nuclear domain^[7,10], revealed that 'safe' HR repair of heterochromatic DSBs requires their relocalization to the nuclear periphery before strand invasion^[8,11] (reviewed in^[4,5]). Relocalization is driven by a striking network of transient nuclear actin filaments (F-actin) assembled at heterochromatic DSBs by the actin nucleator Arp2/3, and extending toward the nuclear periphery^[11] (Fig. 1). Relocalization also requires nuclear myosins (*i.e.*, Myo1A, Myo1B and MyoV) that interact with the heterochromatin repair component Smc5/6, and are activated by Unc45 downstream from Smc5/6, to promote the directed motion of repair sites along actin filaments^[11]. Of note, relocalization is coordinated with HR progression to prevent aberrant recombination. While DSB resection occurs inside the domain^[7], Rad51 recruitment is initially halted by Smc5/6 and dPIAS-dependent SUMOylation^[7,8,12]. Repair restarts at nuclear pores and inner nuclear membrane proteins (the *Drosophila* LINC complex subunits Koi and Spag4) with Rad51 recruitment, likely mediated by a STUbL/RENi dependent degradation or activation

of SUMOylated targets^[8]. Relocalization defects result in unrepaired heterochromatic breaks and widespread chromosome rearrangements in *Drosophila* cells and tissues^[7,8,11-13], revealing the importance of these dynamics to genome integrity. Actin polymerization and myosins also mediate relocalization of heterochromatic DSBs to the periphery of heterochromatic domains (or 'chromocenters') in mouse cells, where Rad51 is recruited^[9,11], revealing conserved pathways.

Similar dynamics contribute to HR repair of other repeated sequences, including rDNA, CAG repeats, and eroded telomeres (reviewed in^[2,3]). In all these contexts, relocalization likely facilitates 'safe' repair by isolating DSBs and their repair templates away from ectopic sequences before strand invasion, or by promoting unconventional repair pathways (reviewed in^[2,3,5]). For example, DSBs in yeast nucleoli relocalize to nuclear pores for HR, which requires Smc5/6 and Rad52 SUMOylation^[14,15]. DSBs in human rDNA relocalize to the nucleolar periphery, with Arp2/3, myosins and the LINC complex mediating these dynamics^[16]. Further, CAG repeats damaged during replication relocalize to nuclear pores in yeast, and this movement relies on the STUbL Slx5/8 and Smc5/6-dependent SUMOylation of the break-induced replication (BIR) components required for fork restart (Rad52, Rad59, and RPA)^[17]. Eroded telomeres also relocalize to nuclear pore complexes in budding yeast, through a process involving STUbL and SUMOylation of telomeric proteins, including RPA^[18]. Finally, in human cells, dysfunctional telomeres relocalize to the nuclear periphery in a nuclear F-actin and nuclear pore-dependent manner^[19]. Together, these studies point to highly conserved pathways for relocalization of DNA breaks in repeated sequences, albeit with differences in terms of SUMOylation targets and final destination of the movement.

A critical discovery from heterochromatin repair studies in *Drosophila*, is that relocalization occurs through directed motions^[11]. These mostly occur between the heterochromatin domain periphery and the nuclear periphery, *i.e.*, where actin filaments assemble, and are consistent with the observation that repair sites slide along the filaments^[11]. In contrast, repair focus motion is mostly sub-diffusive inside the heterochromatin domain and at the nuclear periphery^[5,11], where other constraints to the motion prevail (*e.g.*, phase separation and compaction inside the heterochromatin domain and anchoring structures at the nuclear periphery^[5,8]). These studies and computational simulations further highlighted that in a context of mixed trajectories (*i.e.*, alternating sub-diffusive and directed motions), traditional mean-square displacement (MSD) analyses are insufficient to detect directed motions and more sophisticated methods need to be applied^[11,20] (reviewed in^[1,5]). Application of such methods also revealed directed motions in other contexts, such as for BIR repair of yeast subtelomeric DSBs in yeast, where dynamics are driven by nuclear microtubules and Kar3 kinesin^[21], or for repair of stressed replication forks at the nuclear periphery in human cells, where relocalization requires nuclear F-actin and myosin II^[22]. Additionally, these methods unmasked directed motions for persistent DSBs that move to the nuclear periphery in budding yeast^[21], reversing the initial conclusion that these occur by Brownian/diffusive motion^[23]. These studies point to the importance of applying dedicated tools to identifying directed motions, and suggest

that nuclear filaments and motors might contribute to repositioning repair sites in more contexts than initially thought (reviewed in^[5]).

Multi-scale imaging also revealed that different features of repair focus dynamics are detected at distinct time scales of imaging^[24] (reviewed in^[1]). More frequent imaging (e.g., in the millisecond-scale) detects local chromatin dynamics, such as those driven by Rad51 and homology search, while coarser imaging kinetics (e.g., in the second to minute-scale) detect longer range motions, including directed motions for heterochromatic breaks^[11,24]. Thus, choosing the right the imaging regimen is critical for identifying different types of motion.

Local and global chromatin responses for heterochromatin repair

It is a common misconception that heterochromatin is refractory to protein access and repair because of its silent and compact state. On the contrary, heterochromatin is accessible to large macromolecules and protein complexes, including chromatin remodelers and histone modifiers (reviewed in^[3]). Heterochromatin is also organized as a phase separated domain (see next section), characterized by selective permeability for certain molecules, high diffusion of molecules within the domain, and quick response to post-translational modifications that alter the biophysical properties of the domain^[25,26]. Consistent with an accessible environment, early DNA damage detection and signaling occur efficiently in heterochromatin^[7,27,28], with foci of proteins responding to resection appearing even faster than in euchromatin^[7].

In addition to providing accessibility *per se*, heterochromatin responds dynamically to damage. First, the entire domain expands in mouse and *Drosophila* cells^[7,29], and dynamic protrusions emerge during focus relocalization^[7]. Expansion has been linked to HP1 β phosphorylation by Casein Kinase 2 in mammalian cells^[29], which increases HP1 β turnover on the chromatin^[29]. Blocking this pathway affects H2AX phosphorylation, suggesting a role in DSB signaling^[29]. In fly cells, expansion is dependent on early DSB processing (*i.e.*, resection and ATR-dependent checkpoint activation)^[7], although the targets remain unknown, and the significance of expansion to repair still needs to be understood. Expansion might reflect the loosening of the domain to facilitate relocalization of heterochromatic DBSs, and/or changes in phase separation properties promoting new protein accessibility, focus dynamics, and/or repair progression.

Second, local chromatin changes occur at heterochromatic DSBs and influence repair, similar to observations in other genomic regions^[30,31] (reviewed in^[3,5]) (Fig. 2). For example, in mammalian cells the phosphorylation of the heterochromatin component Kap1 by the checkpoint kinase ATM promotes chromatin 'loosening' and repair progression^[6], while in *Arabidopsis* the ATM-dependent phosphorylation of a heterochromatin-specific H2A variant has been suggested to increase chromatin accessibility^[32]. Recent studies also indicate that in human cells the Snf2-like chromatin remodeler HELLS facilitates HR repair in heterochromatin by promoting the recruitment of the resection component CtIP^[33]. Repair defects after HELLS RNAi can be rescued by treatment

with chloroquine, which relaxes the chromatin, consistent with a role for HELLS in heterochromatin relaxation during repair^[33]. In line with a role for local heterochromatin loosening to facilitate DNA repair, decompaction of mouse chromocenters has also been detected after 405nm^[28] or UV^[34] laser treatments, and has been linked to histone H1 displacement in response to UV damage^[34].

Whether increasing heterochromatin accessibility in response to damage also requires the removal of 'silent' chromatin marks is controversial. Super resolution imaging of heterochromatic regions and studies in response to laser-induced DSBs suggest that H3K9me3 is largely retained during chromatin relaxation at IR or Cas9-induced DSBs targeting the major satellite, in mammalian cells^[29,35]. Similarly, UV-induced heterochromatin relaxation occurs in conditions that maintain H3K9me3 and HP1 α ^[34]. However, ChIP analysis identified a Kdm4A-dependent increase in H3K9me1 and H3K56me1 at site-specific heterochromatic DSBs in *Drosophila*^[36], and Kdm4A is required for relocalization of heterochromatic DSBs^[36,37], suggesting a local reduction of silencing during focus dynamics. This might be a transient response, as H3K9me3 and H3K56me2 also increase at heterochromatic DSBs in this context, which promotes HR over NHEJ^[36].

Together, these studies suggest that local and global heterochromatin decompaction might be coordinated by different mechanisms, including histone modifications, histone loss, post-translational modification of chromatin-associated proteins, and/or chromatin remodeling. The mechanisms responsible remain to be clearly defined across different model systems and repair pathways, and transient responses might require high resolution techniques to be detected.

Other contributors to HR focus dynamics: looping, clustering, and phase separation

Recent studies have shown that spatial organization of chromatin in nuclear subdomains, and damage-induced changes of this organization, are important contributors to local repair dynamics. Here, we highlight recent advances in our understanding of these responses and point out how they might operate in the unique context of heterochromatin.

DNA looping

The genome is organized in a higher order structure defined by topologically associating domains (TADs), whose boundaries are demarcated by CTCF and organized as DNA loops (reviewed in^[38]). Recent studies have established the importance of this organization in regulating γ H2AX spreading, *i.e.*, the phosphorylation of the histone variant H2AX that spans Kb to Mb-sized domains on each side of the DSB and contributes to DSB signaling. Hi-C and super resolution imaging of human cells revealed that γ H2AX mostly spreads within TAD boundaries, identifying TADs as functional units for DSB signaling and repair^[35,39]. Hi-C and ChIP-Seq studies further established that γ H2AX spreading relies on cohesins that induce a one-sided loop extrusion from each

side of the break to the nearest CTCF^[40], and this response appears to be conserved from yeast to humans^[40-42]. Notably, increased chromatin contacts detected within the TAD during γ H2AX spreading, are consistent with the increased mobility of DNA ends observed by live imaging in multiple studies (reviewed in^[1]). Super resolution imaging also identified examples of γ H2AX spanning across multiple TADs. These are organized in 3D circular structures by 53BP1 and Rif1, which maintain local chromatin compaction while preventing DSB hyper-resection^[43].

How the pre-existing topological organization of the genome and damage-induced looping participate in heterochromatin repair is unknown. TADs have been described in pericentromeric regions, at least in single copy sequences^[44,45], and Cohesins, Rif1 and CTCF are enriched in heterochromatin^[46,47], consistent with the organization of 3D structures. However, the presence or organization of TADs in highly repeated DNA sequences, which occupy most pericentric heterochromatin, is not known. Cohesin recruitment to DSBs, γ H2AX spreading mechanisms, and TAD re-organization in response to damage, are also elusive in this domain. Importantly, looping could facilitate relocalization of heterochromatic DSBs to outside the domain and local chromatin changes, while maintaining silencing and compaction in nearby regions. Thus, understanding these mechanisms is important to establish the role of nuclear architecture in heterochromatin dynamics and repair progression.

Focus clustering

Another component contributing to the dynamics of repair foci undergoing HR is focus clustering, *i.e.*, the non-elastic collision of DSB repair sites, which has been detected across different chromatin contexts and model systems (reviewed in^[48,49]). Focus clustering might facilitate DSB signaling and repair progression by increasing the local concentration of repair components^[50], and studies in human cells revealed the importance of clustering in promoting resection of euchromatic DSBs^[50]. Clustering is also observed in HR-prone DSBs in G1 cells, *i.e.*, in the absence of a sister chromatid, where it might induce the sequestration of DSBs in a paused state for subsequent repair in S-phase^[51]. Clustering requires actin nucleation in both *Drosophila* and human cells^[11,50,51], although the underlying mechanism likely differs from that driving directed motion of heterochromatic repair foci. In fact, the myosin activator Unc45 is not required for focus clustering in *Drosophila* euchromatin, while it is necessary for directed motion of heterochromatic DSBs^[11]. Actin filaments might promote clustering by generating propelling forces to move repair sites^[50], or by creating structures along which foci concentrate due to the 'wetting' behavior of phase separated structures^[52] (see next section). Alternatively, they might create nucleoplasmic flow dynamics that increase the probability of collision between repair foci, similar to the role proposed for short nuclear microtubules in Rad52 focus clustering in budding yeast^[52].

Notably, repair focus clustering is also frequently observed inside the heterochromatin domain^[7], where it might facilitate early HR steps, including resection^[7,9]. However, in this context F-actin and myosins do not seem to be

required to promote clustering, and other properties of phase separated domains might be responsible for this response.

Phase separation

Once foci are positioned in close proximity, their fusion can be promoted by the phase separating properties of these structures. Liquid–liquid phase separation (LLPS) is typically established by intrinsically disordered regions (IDRs) of proteins interacting with each other through multivalent weak interactions, which create a local environment with distinct biophysical properties from its surroundings (reviewed in^[53]). At repair sites LLPS appears to be induced by multiple components, including: i) damage-induced long non-coding RNA, which promote the molecular crowding of the largely unstructured DNA repair protein 53BP1^[54,55]; ii) the nucleic acid-mimicking biopolymer poly(ADP-ribose) (PAR), and its associated protein FUS^[56–59]; iii) repair proteins, like yeast Rad52, bacterial SSB (the homolog of RPA), and human TopBP1^[52,60–62]; and iv) chromatin^[63–65], for which the ability to create droplets *in vitro* is dependent on the structurally disordered histone tails, chromatin modifications, and chromatin-associated-proteins^[63,64,66]. Intriguingly, phase separation can also be induced by bridging molecules (also called ‘bridging-induced phase separation’, or BIPS)^[67] and this can be mediated by cohesins at DSBs, although the role of BIPS in damage responses is still unknown. Phase separation might facilitate clustering by increasing the affinity between foci relative to the surrounding environment. In this view, the increased dynamics of repair foci, promoted by either nuclear structures or cytoplasmic forces transmitted to the nucleus through the LINC complex that spans the nuclear membrane, would also promote clustering by increasing the contact probability between repair foci. Notably, phase separated environments are characterized by selective permeability. Thus, in addition to facilitating interactions with other repair foci or nuclear structures, phase separation of repair sites can influence repair pathway choice and promote repair progression by increasing the local concentration of certain repair components while excluding others (reviewed in^[5,53]).

How LLPS of repair foci affects repair in pericentromeric heterochromatin is unknown. Notably, heterochromatin is, *per se*, phase separated^[25,26], which likely influences repair at different levels. First, it might concentrate certain repair components while excluding others, regulating repair progression inside the domain^[5] (Fig. 3). Accordingly, HP1a, which mediates phase separation of *Drosophila* heterochromatin, is required for Smc5/6 enrichment inside the domain and for Rad51 exclusion from the domain^[7]. Also, the early NHEJ component Ku80 is not detectable in the domain^[7], suggesting broad exclusion that might help promote HR^[7,8]. Second, a phase separated environment could facilitate focus clustering inside the heterochromatin domain, promoting early resection. Consistent with this hypothesis, repair focus clustering inside the domain does not depend on Arp2/3^[11], and relocalization of repair sites to outside the domain is frequently concurrent with the splitting of these clusters into smaller foci^[7]. Third, LLPS could promote local chromatin decondensation to facilitate repair. This is consistent with the fact that condensate growth correlates with a reduction in chromatin density, at least in the context of synthetically induced chromatin-associated condensates^[68]. Fourth, LLPS enables fast regulated

changes in biophysical properties, which could in turn facilitate repair progression (reviewed in^[69]). For example, chromatin modifiers or phosphorylation of heterochromatin components might induce expansion/relaxation of the domain, promoting chromatin accessibility to repair proteins and relocalization of repair foci^[7]. Finally, the formation of phase separated repair foci, within the already phase separated heterochromatin domain, would facilitate the free diffusion of repair foci toward the edge of the heterochromatin domain, where they initiate directed motion. In agreement with this idea, the initial phase of focus relocalization from inside the heterochromatin domain to the heterochromatin domain periphery is rarely concurrent with directed motions or visible nuclear actin filaments^[5,11], suggesting that different forces contribute to these dynamics.

SUMOylation and phosphorylation, both of which regulate phase separation and relocalization of heterochromatic repair sites^[7,12], could also operate by modulating LLPS of repair foci. LLPS might affect heterochromatin repair at nuclear pores as well, where intrinsically disordered phenylalanine-glycine-rich nucleoporins (FG-Nups) are known to establish a phase separated environment within the pores^[70]. It is tempting to speculate that repair restart at the pores is influenced by such a local environment, which might retain a high concentration of components required to remove the SUMOylated block to HR progression and promote strand invasion. Thus, phase separation likely influences several aspects of DSB repair in heterochromatin, and understanding how pre-existing biophysical properties and damage-induced changes in this domain contribute to the spatiotemporal regulation of HR repair is an important goal for future studies.

Conclusions and perspectives

Recent studies have revealed that nuclear and chromatin organization and dynamics play crucial roles in HR repair, even in more diverse contexts than initially thought. Understanding the biophysical properties of these motions, how they are regulated, and their impact on repair are important goals and exciting challenges. Pericentromeric heterochromatin and other repeated DNA sequences are particularly dependent on repair dynamics for their stability. While much has been recently learned about the nature of these dynamics, many of the mechanisms involved still need to be understood. Which chromatin changes occur at global vs local scales also requires a deeper investigation. Future work should also clarify how DNA looping and phase separation affect repair dynamics in heterochromatin and other repeated sequences, establish conserved pathways across different organisms, and define how misregulation of these pathways contributes to human diseases. The tools are now in place for exciting new discoveries in the coming years.

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Legends

Figure 1. Model of heterochromatic DSBs relocation pathway.

DSB detection and resection occur inside the heterochromatin domain, where Mre11 and HP1a promote the loading of Arp2/3 and the nuclear myosins. After resection, Smc5/6 complex, its Nse2 SUMO E3 ligase subunits (Cerv and Qjt) and the SUMO E3 Ligases dPIAS are also recruited to heterochromatic DSBs to generate a block to HR progression (*i.e.*, Rad51 recruitment). Repair foci move to the heterochromatin domain periphery, while Scar and Wash activate Arp2/3, inducing actin polymerization at the DSB site. Next, Unc45 recruitment by Smc5/6 activates nuclear myosins that 'walk' along actin filaments while interacting with Smc5/6. This drives the directed motion of repair sites to nuclear pores or inner nuclear membrane proteins (INMPs) where the SUMO-targeted Ubiquitin Ligase (STUbL)/RENi complex stabilizes the association of repair sites with the nuclear periphery. STUbL also ubiquitylates SUMOylated proteins, inducing their proteasome-mediated degradation or activation (not shown), thus enabling Rad51 recruitment and HR progression. Relocalization prevents ectopic recombination and promotes 'safe' repair, by isolating damaged sites and their homologous templates (grey lines) from undamaged heterochromatic repeats before strand invasion.

Figure 2. Model for the local chromatin changes following DSBs at heterochromatic sites.

The compact state of heterochromatin (top) undergoes local changes in response to DSBs (bottom) that facilitate repair. Chromatin remodeling and relaxation are orchestrated by chromatin modifiers, including Kap1 and HELLS in human cells and Kdm4A in *Drosophila*. Histone modifications occurring in heterochromatin might include: i) phosphorylation of H2A variants by checkpoint kinases; ii) increased H3K9me1 and H3K56me1, likely followed by or concurrent with and H3K9me2/3 and H3K56me2/3 increases, iii) histone loss. Low levels of HP1a at the repair sites could also facilitate chromatin loosening and repair progression. These local changes might facilitate HR repair by promoting the recruitment of repair factors, affecting repair pathway choice, and increasing dynamics, promoting repair progression.

Figure 3. Model for clustering and phase separation of heterochromatin repair foci.

Phase separation of the heterochromatin domain and repair foci might influence repair at different levels, by: i) increasing the concentration of early HR repair components (*i.e.*, damage signaling proteins and resection components) while exclude others (*i.e.*, Rad51, Ku80); ii) promoting focus clustering inside the domain; iii) promoting chromatin decondensation; and iv) facilitating the free diffusion of repair foci to the edge of the heterochromatin domain, where directed motions along actin filaments start. Phase separation of nuclear pores might also create an environment with different biophysical properties, which is compatible with Rad51 access and repair progression.

Graphical Abstract. Nuclear dynamics following DSB at heterochromatic sites.

Following DNA damage, early DSB detection and signaling occur efficiently inside the heterochromatin domain (1). The phase separating properties of repair foci inside a phase separated heterochromatin domain can facilitate focus clustering (2), focus diffusion toward the heterochromatin domain periphery (3), and the spatial and temporal regulation of HR repair. Meanwhile, the entire domain expands (4) while local chromatin changes (5) promote chromatin unfolding to promote repair and dynamics. TADs might organize functional units (loops) for DSB signaling and repair (6). Nuclear actin (F-actin) (7) and myosins drive the directed motion of heterochromatic repair site to the nuclear periphery, where they are anchored and HR repair continues (8) in a 'safe' environment. ONM: Outer Nuclear Membrane; INM: Inner Nuclear Membrane.

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Figure 1

Figure 2

Figure 3