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Review Article

Actin' between phase separated domains for heterochromatin repair☆,☆☆

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ABSTRACT

DNA double-strand breaks (DSBs) are particularly challenging to repair in pericentromeric heterochromatin because of the increased risk of aberrant recombination in highly repetitive sequences. Recent studies have identified specialized mechanisms enabling 'safe' homologous recombination (HR) repair in heterochromatin. These include striking nuclear actin filaments (F-actin) and myosins that drive the directed motion of repair sites to the nuclear periphery for 'safe' repair. Here, we summarize our current understanding of the mechanisms involved, and propose how they might operate in the context of a phase-separated environment.

1. Heterochromatin repair challenges

Studies across different organisms have revealed that genomes are hierarchically organized into distinct domains, from local loops, to higher level topologically-associating domains (TADs), and large chromosome territories (reviewed in [1,2]). On different scales, domains represent regions of higher frequency contacts, while inter-domain interactions are more rare and highly regulated [1,2]. Components maintaining the nuclear organization in domains include: CCCTC-binding factor (CTCF) and cohesins that organize TADs [3–5]; the lamina, which stabilizes specialized TADs named lamina-associated domains (LADs) [6]; and the nucleolus, which organizes nucleolus-associated domains [7,8] (NADs) [9]. Additional interactions are transiently established at transcription or replication 'factories' [10–12]. Biophysical properties of phase-separated domains provide further constraints to the movement of genomic sites, *e.g.,* in pericentromeric heterochromatin [13,14], nucleoli [15], nuclear pores [16], and repair sites [15]. One of the most exciting challenges in recent years has been understanding what forces promote intra- and inter-domain movements for different functions like DNA replication, transcription, and repair.

One of the largest and better described phase-separated nuclear domains is pericentromeric heterochromatin [13,14] (hereafter 'heterochromatin'), which accounts for about 30% of fly and human genomes [17–19], and is absent in budding yeast. Heterochromatin is characterized by 'silent' histone marks (*e.g.,* H3K9me2/3), and associated proteins such as heterochromatin protein 1 (*e.g.,* HP1a in flies [20,21] and HP1α/β in mammalian cells [22,23]), which contribute to its compaction and phase separated state [13,14] (reviewed in [24]). Notably, heterochromatin is functionally and structurally distinct from LADs distributed along the chromosome arms, and in contrast to those, it is not usually associated with the nuclear periphery (see for example [9,25–30], reviewed in [31]). Heterochromatin is mostly composed of repeated DNA sequences. In *Drosophila*, about half are 'satellite' repeats (predominantly 5-base pair sequences repeated for hundreds of kilobases to megabases) and the rest are transposable elements, scrambled repeats, and about 250 isolated genes [17–19]. The abundance of repeated sequences in heterochromatin poses unique challenges to DSB repair and genome stability [27,31–33].

The two main pathways for DSB repair are non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is characterized by direct re-joining of the two ends, which frequently generates small mutations at the repair site [34]. HR initiates with resection to form single-stranded DNA (ssDNA), which invade 'donor' homologous templates for DNA synthesis and repair [35]. In single-copy sequences, a unique donor is present on the sister chromatid or the homologous chromosome, and HR is largely 'error free' [35]. In heterochromatin, however, the availability of up to millions of potential donor sequences associated with different chromosomes can initiate

Abbreviations: DSBs, double-strand breaks; HR, homologous recombination; NHEJ, non-homologous end joining; TADs, topologically associated domains; NADs, nucleolus-associated domains; LADs, lamina-associated domains; ssDNA, single-stranded DNA; STUbL, SUMO-targeted ubiquitin ligase; SUMO, small ubiquitin-like modifier; MSD, mean square displacement; IDPs, Intrinsically disordered proteins; BIR, break-induced replication; FG-nups, phenylalanine-glycine rich nucleoporins; F-actin, filamentous, polymeric actin; G-actin, globular, monomeric actin

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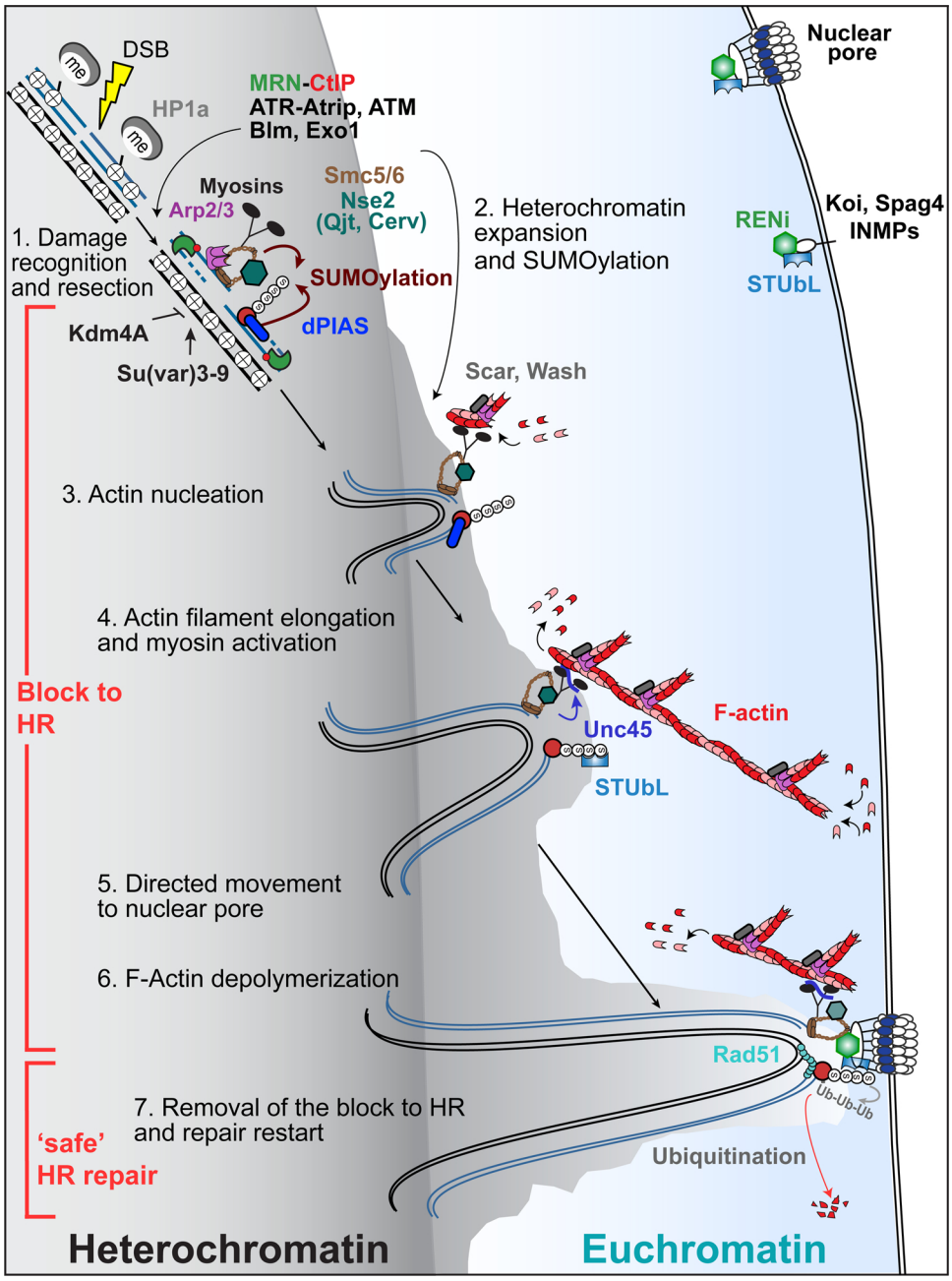

Fig. 1. Model of the molecular mechanisms for 'safe' HR repair of *Drosophila* heterochromatin: DSB detection and resection occurs efficiently inside the heterochromatin domain, while Kdm4A and Su(var)3-9 contribute to repair pathway choice (1). Checkpoint kinases (ATR, ATM) and resection components (Mre11 complex MRN-CtIP, Blm, Exo1/Tosca) facilitate heterochromatin expansion (2), while Mre11 and HP1a promote the recruitment of Arp2/3 and myosins to repair sites. Smc5/6 subunits Nse2/Qjt and Nse2/Cerv, and dPIAS, block Rad51 recruitment inside the heterochromatin domain via SUMOylation, while recruiting the myosin activator Unc45. Scar and Wash activate Arp2/3, inducing actin polymerization toward the nuclear periphery (3–4). The myosin-Smc5/6 complex associated with damaged DNA translocates along actin filaments with directed motions (5), and anchors repair sites to nuclear pores or INMPs *via* STUbL-RENi proteins. At the nuclear periphery, STUbL might promote Rad51 recruitment *via* ubiquitination and proteasome-mediated degradation of SUMOylated proteins, and 'safe' repair with the sister chromatid or the homologous chromosome that relocalized in concert with the damaged DNA (7). Local chromatin changes might contribute to relocalization and repair progression (not shown). Actin filaments are highly dynamic and disassemble during and after relocalization (6).

unequal sister chromatid exchanges or intra-/inter-chromosomal recombination, leading to deletions, duplications, translocations, release of extra-chromosomal DNA circles (ECCs), and formation of dicentric or acentric chromosomes [26–28,36–40]. Despite this danger, HR is a primary pathway to repair heterochromatic DSBs in *Drosophila* and mammalian cells [26,28,30,38,39,41–43], and specialized mechanisms enable 'safe' HR in heterochromatin while preventing aberrant recombination.

2. Choreography of heterochromatin repair mechanisms

Many of the molecular mechanisms responsible for heterochromatin repair have been initially characterized in *Drosophila* cells, where the organization of heterochromatin in a distinct nuclear domain greatly facilitates cytological approaches [9,21,26]. The recruitment of repair components to DSBs also results in cytologically visible foci, which can be detected in the nucleus using live and fixed cell imaging [26,44,45]. These studies have revealed that HR repair is tightly regulated in space and time (Fig.1): proteins required for resection are recruited to repair sites inside the domain, while recruitment of strand invasion components is temporarily halted [26,28,38]; next, the heterochromatin domain expands and repair sites move to the nuclear periphery, where HR progresses [26,28,39]. Inactivating this pathway results in defective heterochromatin repair and aberrant recombination among repeated sequences, revealing its importance to genome integrity [26,28,36–40]. Relocalization likely promotes 'safe' repair by isolating DSBs and their repair templates away from ectopic sequences before strand invasion [27,31,33,46]. Notably, *Drosophila* homologous chromosomes are paired in interphase [47], and accordingly both sister chromatids and homologous chromosomes can provide repair templates in this system [39,43]. Similar dynamic responses occur in mouse cells [27,30,39,48,49], where heterochromatin is organized in several 'chromocenters' [50], suggesting conserved mechanisms for heterochromatin repair [31,33]. Here we provide an overview of

heterochromatin repair steps in *Drosophila* cells, conserved pathways in mammalian cells, and interesting discoveries in plants. We will also point out some of the most important unanswered questions in the field.

2.1. DSB detection and signaling

In response to ionizing radiation (IR), DSB detection and signaling occurs promptly in heterochromatin [26,30,39,49]. In *Drosophila*, foci of γH2Av (an early mark of DSB formation, corresponding to mammalian γH2AX [51]) and Mdc1/Mu2 (a signaling components that binds to γH2Av [52]) form within seconds to minutes from IR [26,40,52], and with kinetics surprisingly similar to those in euchromatin [26]. Intriguingly, foci of proteins marking resected DNA (*e.g.,* ATRIP and TopBP1) form even faster and appear brighter in heterochromatin than in euchromatin [26], suggesting that either resection or focus clustering (*i.e.*, the non-elastic collision between repair foci [27]) is more efficient in heterochromatin [26]. In mouse cells, damage recognition and processing also occur inside the heterochromatin domain, with the formation of γH2AX and RPA foci [27,30,39,49]. By revealing high efficiency of early repair progression in heterochromatin, these studies reversed the early assumption that silencing or compaction of heterochromatin imposes a barrier to repair initiation.

Notably, focus clustering might facilitate DSB signaling and repair progression by increasing the local concentration of repair components [27,53]. Studies in mouse cells suggest that clustering promotes resection, at least in euchromatin [53]. Focus clustering is also frequently observed inside the heterochromatin domain [26], and might facilitate early HR steps in this context. Resection is needed for relocalizing heterochromatic DSBs in both *Drosophila* and mouse cells [26,30], and having efficient resection might provide a signal for rapid relocalization of heterochromatic DSBs, preventing accidental strand invasion of ectopic sequences. However, more studies are needed to understand the efficiency of resection in heterochromatin, the mechanisms responsible for focus clustering and resection in this domain, and the importance of both in the spatial and temporal regulation of heterochromatin repair.

2.2. Heterochromatin expansion

Resection and checkpoint activation (particularly ATR [26]) are required for global expansion of the heterochromatin domain in *Drosophila* cells, which starts minutes after IR [26]. This corresponds to an increase of up to 50% in domain size [26,40], and is followed by the formation of dynamic protrusions from the domain during focus relocalization [26]. Expansion might reflect global heterochromatin relaxation to facilitate damage processing or dynamics [26]. In agreement, proteins required for expansion also mediate DSB signaling and relocalization [26]. Heterochromatin relaxation also occurs in mammalian cells [30,48,54], where it has been linked to HP1β T51 phosphorylation by casein kinase 2 (CK2) [48]. Blocking this pathway affects H2AX phosphorylation, revealing its importance in DSB signaling [48]. In *Arabidopsis*, expansion following heterochromatic damage generates 'hollow' chromocenters with repair sites in the center, still isolating repair sites from the bulk of repeated sequences [55].

Of note, global heterochromatin expansion likely facilitates relocalization, but is not sufficient for relocalization to proceed. In fact, relocalization defects have even been observed in conditions when expansion is normal (*e.g.,* after Nse2/Qjt RNAi in *Drosophila* cells) [38], genetically separating heterochromatin expansion from relocalization. Together, more studies are needed to understand the functions of global expansion in heterochromatin repair, along with chromatin changes promoting these responses in different organisms.

2.3. Block to Rad51 recruitment inside the heterochromatin domain

Recruitment of the strand invasion component Rad51 only occurs after relocalization of heterochromatic repair sites to the nuclear periphery in *Drosophila* cells [26,28]. The initial block to HR progression is dependent on Su(var)3-9 and HP1a [26], revealing the importance of silencing in heterochromatin protection during repair. The block also requires SUMOylation by three SUMO E3 ligases: dPIAS and the Smc5/6 subunits Nse2/Qjt and Nse2/Cerv [26,28,38]. Smc5/6 recruitment to heterochromatin relies on HP1a [26], revealing a role for Smc5/6 in heterochromatin protection downstream from HP1a. Removing these components results in aberrant recombination in heterochromatin and widespread chromosome rearrangements [26,28,38]. Rad51 is also recruited after relocalization to outside the chromocenters in mouse cells [30], but relocalization appears to end at the heterochromatin domain periphery [30,39,49], which might provide a functionally isolated environment similar to the nuclear periphery in *Drosophila* cells. Additionally, losing Smc5/6 does not result in Rad51 foci inside mouse chromocenters [30], suggesting alternative or redundant mechanisms to block HR progression in this context. Together, these discoveries revealed the importance of silencing and SUMOylation in blocking Rad51 recruitment inside the heterochromatin domain to prevent aberrant recombination between heterochromatic repeated sequences. The targets of this regulation remain unknown.

2.4. Relocalization mechanisms

Smc5/6 and SUMOylation are also required for relocalizing heterochromatic DSBs to the nuclear periphery in *Drosophila* cells, and recent studies revealed some of the components mediating these dynamics. Relocalization relies on a striking network of nuclear actin filaments (F-actin) that start assembling at repair sites *via* Arp2/3 recruitment [39]. Relocalization also requires Myo1A, Myo1B, and MyoV nuclear myosins, and myosin's ability to 'walk' along the filaments [39]. Notably, Arp2/3 and myosins are recruited to DSBs independently from Smc5/6 [39]. However, Smc5/6 interacts with these components during repair [39], suggesting a regulatory role for this interaction. Intriguingly, Arp2/3 and actin are known SUMOylation targets [56,57], and it will be important to establish the role of Nse2- and dPIAS-dependent SUMOylation in their activity during heterochromatin repair. Smc5/6 is also required for the recruitment of the myosin activator Unc45 to DSBs, suggesting Unc45 as a molecular switch that activates myosins downstream from Smc5/6.

By interacting with both DSBs and myosins [39], Smc5/6 might also provide a physical link between resected DNA and transport mechanisms, translating myosin-driven pulling forces into repair focus movement. Recruitment of Arp2/3 and myosins to repair sites requires the early DSB signaling and processing factor Mre11, and the heterochromatin protein HP1a [39], suggesting the combination of these components as a mechanism for targeting the relocalization machinery specifically to heterochromatic DSBs. Downstream from Mre11, other repair/checkpoint components might mediate Arp2/3 and myosin recruitment, and this still needs to be determined. Together, these data support a model where nuclear F-actin assembles at heterochromatic DSBs to guide their relocalization to the nuclear periphery *via* myosin-driven 'walk' along actin filaments. Arp2/3, actin polymerization, and myosins are also required to relocalize and repair heterochromatic DSBs in mouse cells [39], suggesting conserved pathways.

2.5. Local chromatin changes

Heterochromatin is characterized by a unique chromatin environment, including high levels of H3K9me2/3, H3K56me3, H4K20me3, and H3K64me3 [58–60], which likely influence repair responses in this domain. How this environment contributes to repair and is affected by DSB formation is just starting to emerge. Studies at I-SceI induced site-specific DSBs using the repair cassette *DR-white* in flies, support the model that H3K9me3 and H3K56me3 increase at heterochromatic DSBs to promote HR repair [61]. The histone demethylase Kdm4A

counteracts this response by increasing H3K9me1 and H3K56me1, and favoring NHEJ [61]. Kdm4A is also required for relocalization of heterochromatic DSBs in *Drosophila* cells [62], and this function might be independent from its role in NHEJ, given that NHEJ inactivation does not affect focus relocalization [26]. An interesting possibility is that Kdm4A promotes relocalization by increasing local or global chromatin mobility through a local reduction of silencing marks. In agreement with this, imaging studies show low levels of HP1a at HR repair foci [26], suggesting HP1 is removed, or heterochromatin is loosened, to enable repair progression in *Drosophila* cells.

Additional studies in mammalian cells support this local chromatin 'loosening' model. Specifically, 53BP1-dependent recruitment of Kap1pS824 to repair sites promotes Chd3 release from chromatin, chromatin relaxation, and heterochromatin repair downstream from γH2AX [42,63–66]. Notably, blocking Kap1pS824 does not impair relocalization of DSBs but it affects heterochromatin repair [25,30], consistent with a later function of chromatin relaxation in DSB processing. Kap1pS824 might also play a role in global heterochromatin expansion, given that this modification has been linked to a large-scale increase in chromatin accessibility [67]. *Arabidopsis* does not have Kap1, but the ATM-dependent phosphorylation of the heterochromatin-specific H2A variant H2A.W.7, has been proposed to facilitate chromatin accessibility during repair [68]. While the molecular details remain to be established for Kdm4A, Kap1pS824, and H2A variants in repair pathway choice, repair progression, and dynamics, these studies have begun unraveling heterochromatin-specific changes for DSB repair. A general model is that heterochromatin loosening facilitates early and late heterochromatin repair steps through the regulation of distinct chromatin components.

2.6. Nuclear periphery anchoring and repair progression

In *Drosophila* cells, DSBs move to nuclear pores or inner nuclear membrane proteins (INMPs) of the SUN family Koi and Spag4, where Rad51 is recruited and repair continues [28]. Interaction with the pore is mediated by the 'Y complex' subunit Nup107 [28]. In the absence of these anchoring structures, damaged sites continue exploring the nucleoplasm, eventually returning to the heterochromatin domain [28]. This results in defective heterochromatin repair and gross chromosomal rearrangements [28], revealing the importance of DSB anchoring for 'safe' HR progression. Anchoring also appears to be mediated by the SUMO-targeted ubiquitin ligase (STUbL) Dgrn and its partner dRad60 of the RENi (Rad60-Esc2-Nip45) family protein [28], which are enriched at nuclear pores and INMPs [28]. Dgrn and dRad60 also physically interact with Smc5/6 in response to damage, suggesting that the three components establish a docking complex for repair sites at the nuclear periphery [28].

What restarts repair at the nuclear periphery remains unclear, but a likely possibility is that STUbL proteins ubiquitinate SUMOylated targets for proteasome-mediated degradation [66,69–73] or protein activation [74], removing the SUMOylated block to HR progression. This model predicts that the compartmentalization of SUMOylation activities inside the heterochromatin domain and ubiquitination activities at the nuclear periphery are needed for spatial and temporal regulation of repair.

STUbL (and not RENi) is enriched at heterochromatic DSBs even before relocalization [38] suggesting additional, still unidentified, functions of STUbL in early steps of heterochromatin repair. Consistent with this idea, artificial tethering of the STUbL subunit Slx5 to repair sites in budding yeast is sufficient to target a persistent or unrepairable DSB to the nuclear periphery [75], while recruitment of the STUbL RNF4 to repair sites promotes early DSB signaling in human cells [70].

Of note, RNAi depletion of Arp2/3, myosins, Unc45, STUbL/RENi proteins, nuclear pores, or INMPs, affects relocalization of heterochromatic DSBs without altering the block to HR progression inside the heterochromatin domain, as Rad51 foci do not form inside the domain in these conditions [28,39]. Conversely, losing Smc5/6 or SUMOylation results in Rad51 foci inside the heterochromatin domain [26,28,38], revealing a separation of function between the pathway that blocks HR progression and the mechanism of relocalization. SUMOylation is required for both, but motor or nuclear periphery components only mediate relocalization or anchoring to the nuclear periphery [26,28,38].

These studies also highlighted several distinct functions of silencing histone marks and associated proteins in heterochromatin repair. In *Drosophila* cells, HP1a is required to: i) prevent abnormal Rad51 recruitment inside the domain *via* Smc5/6 and SUMOylation [26,28,38]; ii) promote relocalization of DSBs to the nuclear periphery *via* Arp2/3 and myosin recruitment [39], Smc5/6- and SUMO-dependent Unc45 loading [39], and Kdm4A recruitment [62]; and iii) facilitate nuclear periphery anchoring *via* Smc5/6-associated STUbL-RENi proteins [28]. Additionally, Su(var)3-9-dependent histone methylation facilitates HR repair while Kdm4A-dependent demethylation promotes NHEJ [61]. In mammalian cells, HP1β or Kap1 post-translational modification appears to facilitate heterochromatin loosening, repair, and dynamics [25,48,63]. These studies establish a new paradigm where heterochromatin components promote several steps of heterochromatin repair, rather than interfering with it.

3. Alternative repair pathways in heterochromatin

Studies in *Drosophila* and mammalian cells reveal that, despite the risks of aberrant recombination, heterochromatin is preferentially repaired by HR when both HR and NHEJ are available (*i.e.*, in S and G2 phases of the cell cycle [26,30,41,42]). However, *Drosophila* tissues enriched for G1 cells, and mammalian cells in G1/G0, also largely use NHEJ in heterochromatin [30,41,43,61]. Surprisingly, single-strand annealing (SSA) that is potentially engaged in repeated sequences [76] does not significantly contribute to heterochromatin repair, at least when repair outcomes are characterized with a DR-*white* repair cassette in flies [43]. Further, NHEJ repair occurs inside the heterochromatin domain in mouse cells [30], suggesting that NHEJ progression does not require relocalization. However, heterochromatic DSBs are frequently detected outside heterochromatin domains in *Drosophila* tissues, albeit NHEJ prevails in this context [43], suggesting relocalization can occur during NHEJ, at least in flies. Determining how different heterochromatic DSBs are directed toward distinct repair pathways, and relocalization mechanisms linked to them, remain important open questions in the field.

4. Nuclear F-actin functions and regulation for heterochromatin repair

Actin filaments (F-actin) are major components of the cytoskeleton responsible for cell movement and adhesion, or transport of RNAs and vesicles *via* myosin motors [77–79]. In the nuclei, F-actin functions have long remained elusive because the more abundant cytoplasmic signal interferes with detection of nuclear filaments using traditional staining approaches [80,81]. With recent advances, including the development of nuclear F-actin-specific fluorescent probes [39,80,82–84] (Fig. 2A,B) and techniques to specifically and selectively inactivate nuclear actin polymerization [39,82–85], several nuclear F-actin functions have started to emerge in different cell types. These studies suggest a model where nuclear F-actin is mostly stimulus-driven, is highly dynamic, and mediates chromatin responses to different stresses [46,86]. Functions of nuclear F-actin have been linked to transcription regulation [82,84,85,87,88], mitotic exit [85], centromere maintenance [89], replication origin activation [90], replication fork rescue [91], virus mobilization [92–94], T-cell activation [88] and DSB repair [39,53,83,95,96,147] (reviewed in [46,86]).

During *Drosophila* heterochromatin repair, nuclear F-actin starts polymerizing at repair sites, with most filaments elongating from the

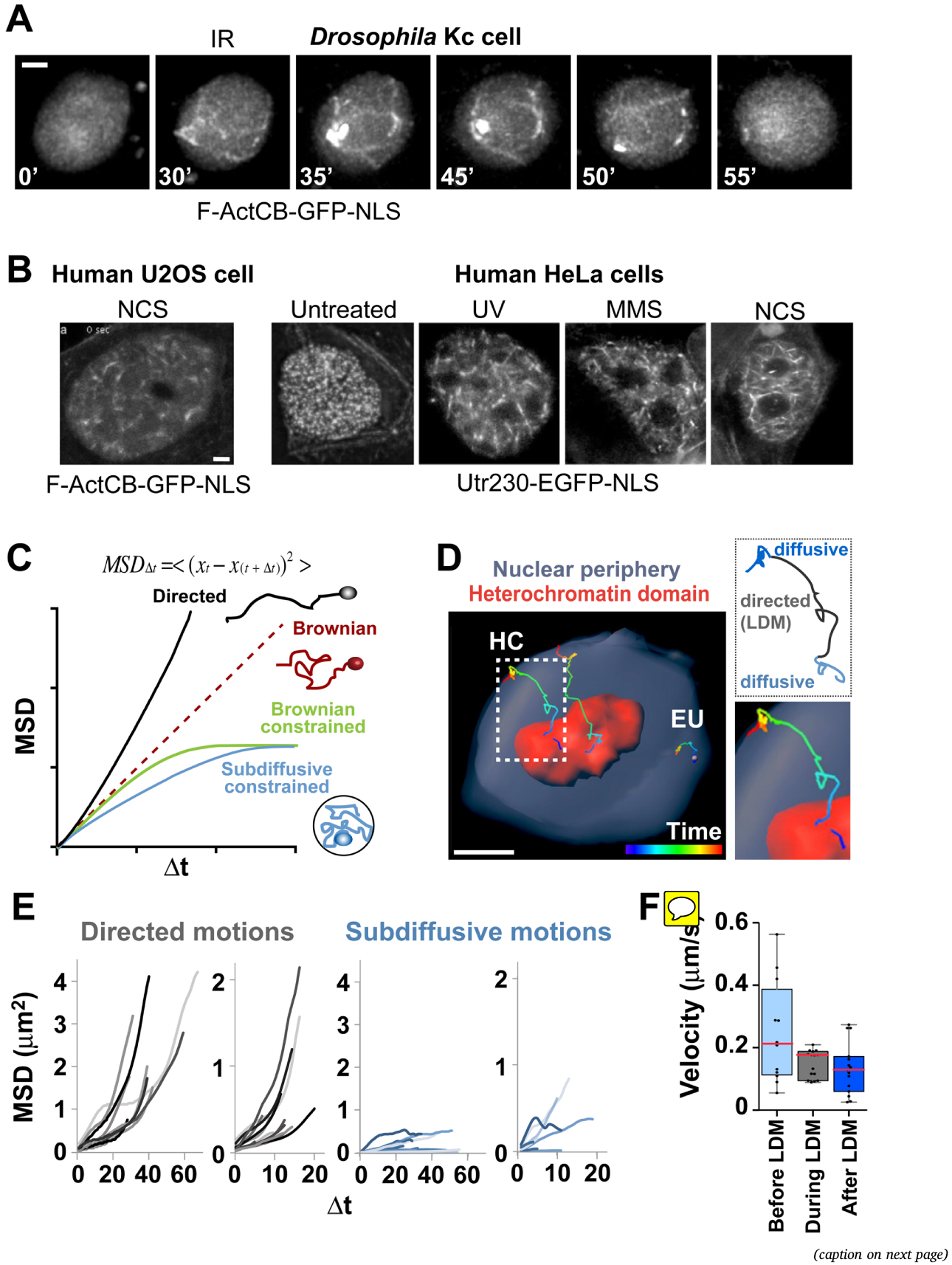

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Fig. 2. Damage-induced nuclear actin filaments generate directed motions for relocalization of heterochromatic DSBs. A–B) Examples of damage-induced nuclear F-actin in indicated cell types and damage treatments (adapted from [39,53,83]). The nuclear F-actin probe chromobody (F-ActCB-GFP-NLS) or utrophin (Utr230-EGFP-NLS) was used as indicated, in either live U2OS [53] and Kc cells [39], or in fixed HeLa cells [83]. In A) times are min after exposure to 5 Gy X-rays. 0′ is before IR. In B) treatments were: 50 pg/ml for 2h or 500 ng/ml for 1 h neocarizinostatin (NCS), 50 J/m^2 UV, or 0.01% methyl methanosulfonate (MMS) for 2 h. C) MSD curves for different types of motion (adapted from [97]). D) Example of a 3D reconstruction and tracking with Imaris of a *Drosophila* cell and heterochromatic (HC) or euchromatic (EU) repair foci, shows track intervals characterized by diffusive or directed motion for heterochromatic repair foci that reach the nuclear periphery (adapted from [97]). E) Time points characterized by directed and sub-diffusive motions were detected with an automated method [97], and confirmed by MSD calculations within those time intervals (adapted form [39]). F) Whiskers plot show the quantification of the speed of focus movement before, during, and after LDMs [39], as indicated (average values are shown in red). The average speed for each tract length was calculated using Imaris. Images reproduced with permissions from Springer Nature. Scale bar = 1 μm.

heterochromatin domain periphery to the nuclear periphery as branched structures [39,148] (Fig. 2A). Repair sites 'slide' along the filaments with directed motions [39,97], consistent with a role of filaments as 'highways' for relocalization. Class I and V myosins (including Myo1A, Myo1B, and MyoV) typically move toward the (+) or 'barbed' end of an actin filament [98,99], corresponding with the nuclear periphery side [39]. While different myosins are involved and whether more than one myosin operates at each repair site remains do be determined.

Actin polymerization and relocalization of heterochromatic repair sites specifically require the actin nucleator Arp2/3, while the nucleators Spire and the formin Dia do not contribute to these dynamics [39]. Additionally, relocalization requires the Arp2/3 activators Scar and Wash, and not Wasp or Whamy [39]. The use of specific nucleators might reflect the ability of the DNA repair machinery to recruit certain components and not others, and relate to the need for filaments with a specific structure. However, more studies are needed to establish the fine structure of these filaments, the significance of 'branches' associated with them, and the regulatory mechanisms coordinating actin polymerization with DSB relocalization and repair. Why polymerization mostly occurs outside the heterochromatin domain is also unknown, particularly given that Arp2/3 is already present at heterochromatic repair sites before relocalization [39,148].

Damage-induced actin filaments are also highly dynamic. Heterochromatin-associated structures in *Drosophila* frequently elongate and shrink, and disassemble after relocalization of repair sites [39] (Fig. 2A). It is still unclear what signals and actin remodelers regulate these dynamics, and what are their roles in repair progression, but filament dynamics might enable 'probing' the crowded nuclear space for an efficient relocalization path.

Additionally, release of monomeric actin during filament disassembly might affect repair progression through the contribution of G-actin in chromatin remodeling. Several chromatin modifiers contributing to DSB repair contain monomeric actin (G-actin) (*i.e.,* HDAC1/2, Tip60, INO80, SWR1, SWI/SNF and RSC; reviewed in [100]), which is critical for their assembly, integrity and function [100]. While the roles of these chromatin modifiers in heterochromatin repair remains to be established, it is possible that G-actin release during depolymerization contributes to assembling and engaging these components during repair.

In addition to relocalizing heterochromatic DSBs in *Drosophila* cells, nuclear F-actin has been proposed to drive local dynamics for focus clustering in human cells, promoting HR repair in euchromatin [53] (reviewed in [46]). Arp2/3 is enriched at DSBs and required for repair focus movement [53], and in this context actin assembles short and highly dynamic structures (Fig. 2B) tracking with HR sites [53]. Arp2/3 also mediates clustering of euchromatic foci in *Drosophila* cells [39], suggesting conserved pathways. Actin structures might promote clustering by generating propelling forces to move repair sites [53], although more studies are required to understand how F-actin works in this context.

Notably, the dynamic movement of human repair sites requires Wasp [53], revealing a distinct mechanism for Arp2/3 activation than that operating in *Drosophila* heterochromatin. Additionally, the myosin activator Unc45 is not required for focus clustering in *Drosophila* euchromatin [39], revealing that the mechanisms responsible for relocalization of heterochromatic DSBs and for clustering of euchromatic breaks are genetically distinct.

Formins and Spire proteins have also been identified as actin nucleators in response to different DNA damaging agents [83] (Fig. 2B), and for focus clustering in G1 [101], suggesting that the distinct nucleators might contribute to damage-induced F-actin assembly in different contexts of repair, cell cycle phase, chromatin, or cell type (reviewed in [46]).

Together, these studies identified two separate functions of nuclear F-actin in DSB repair. In heterochromatin, F-actin and myosins enable the relocalization of heterochromatic DSBs after resection and Smc5/6 recruitment, to prevent aberrant recombination between repeated sequences and enable 'safe' HR repair at the nuclear periphery. In euchromatin, actin polymerization promotes DSB movement, clustering and resection in a myosin-independent fashion. The structure of F-actin in different contexts might reflect the different functions. For example, short actin polymers might be sufficient for local dynamics mediating clustering; while long filaments might be needed for the myosin-dependent, longer-range, directional motions of heterochromatic DSBs. More studies are required to characterize these structures and nucleating mechanisms in different cell types, cell cycle phases, chromatin, and repair contexts.

5. Directed motion of repair sites

One of the most important discoveries so far from heterochromatin repair studies is that focus movement is characterized by directed motion [39,97], similar to F-actin and myosin-driven movements in the cytoplasm [102]. A traditional approach to distinguishing Brownian *versus* directed motion is the mean-square displacement (MSD) analysis of the positional data for repair sites [97,103]. When MSD values are plotted at increasing time intervals, graphs with a progressively increasing slope describe directed motion, while graphs showing a linear dependence indicate Brownian motion [97,103] (Fig. 2C). Chromatin is also subject to constraints due to its polymeric nature, compaction, molecular crowding, and anchoring to nuclear structures, resulting in subdiffusive rather than Brownian motion, and flattened MSD curves [97,103,104]. In addition, when subdiffusive motions occur in a confined space (*e.g.,* the nucleus or a phase-separated domain), MSD graphs reach a plateau proportional to the confinement radius [97,103].

However, when directed motions alternate with diffusive motions (mixed trajectories [97]), and initiate asynchronously in the population of foci, they cannot be detected with a simple MSD analysis [46,97,39]. New analytical methods have been developed to uncover tracts of directed motions in the context of mixed types of motion [97]. These analyses revealed that each heterochromatic repair site leaving the heterochromatin domain undergoes long-lasting directed motions (LDMs) (Fig. 2D,E), and those typically last about 24 min, consistent with the average duration of nuclear actin filaments [39]. Remarkably, directed motions of heterochromatic repair sites mostly occur between the heterochromatin domain periphery and the nuclear periphery [39], *i.e.,* where most nuclear actin filaments are organized [39,148]. Inside the heterochromatin domain and until foci reach the periphery of the domain, the movement is largely subdiffusive confined [39] (Fig. 2D,E),

likely because heterochromatin compaction [105] and phase separation [13,14] limit dynamics. Similarly, after relocalization, focus movement is highly confined by nuclear periphery anchoring [28,39]. Notably, the average speed of focus motion does not increase during directed motions (Fig. 2F), suggesting that the myosin-driven movement along actin filaments does not enhance focus speed. Rather, it might provide directionality and counteract other forces that limit the release of repair foci from the heterochromatin domain (*e.g.,* chromatin compaction and phase separation).

Application of similar analytical methods [91,106] revealed directed motions associated with subtelomeric DSBs repaired by the HR sub-pathway break-induced replication (BIR) in *S. cerevisiae* [106], and with damaged replication forks in human cells [91] (reviewed in [46]). Additionally, these methods unmasked directed motions for persistent DSBs that move to the nuclear periphery in budding yeast [69,107–110], reverting the initial conclusion that those are characterized by Brownian/diffusive motion [111]. Directed motions have also been detected during homology search for HR repair of telomeres in ALT cells [112]. These studies point to the importance of applying dedicated tools to identifying directed motions, and suggest that nuclear structures and motors might contribute to repositioning repair sites in more contexts than initially thought, including where diffusive motions appear to prevail: DSBs in rDNA [113–116,147], damaged telomeric and subtelomeric sequences [112,117–119], damaged replication forks in yeast [69,120], homology search in different contexts [121,122], chromosome territory repositioning [123,124], and focus clustering [26,27,39,53,101,125–130] (reviewed in [31,33]).

Additionally, while studies in *Drosophila* and mammalian cells identified nuclear F-actin and myosins responsible for directed motions [39,91], relocalization of subtelomeric sites for BIR repair in yeast has been linked to nuclear microtubules and the kinesin Kar3 [106], suggesting that nuclear architecture and motor components contributing to repair dynamics might be distinct across different cell types and repair pathways. Also in this context, loss of Kar3 does not affect the average speed of motion [106], suggesting a role for filaments and motors in providing a directionality to the repair site rather than affecting speed. More studies are needed to identify repair contexts relying on directed movements and the structural/motor components mediating these dynamics.

6. HR regulation in phase separated environments

A critical element for successful heterochromatin repair is the ability to separate repair steps in space and time, thus enabling repair progression only at the nuclear periphery. Compartmentalization of repair activities in the nucleus is a likely mechanism to explain this spatial and temporal regulation. For example, the enrichment of HP1a and SUMOylating proteins inside the heterochromatin domain [26,28], and anchoring of SUMO-binding/processing proteins and proteasomes to nuclear pores [28,69,131–133], explain at least some aspects of this regulation. However, how this compartmentalization is achieved is only partially understood, and the recent characterization of the heterochromatin domain as a phase-separated environment provides further insights to understanding this regulation.

Studies in *Drosophila* and mammalian cells revealed that HP1 molecules establish a phase transition compartment through a liquid-like HP1 population that surrounds the chromatin-bound fraction [13,14]. This function is in addition to the ability of HP1 to generate a compact chromatin state through HP1-HP1 interactions of chromatin-bound HP1 molecules [13,14]. Unlike chromatin compaction, phase separation provides a mechanism for selective accessibility of the heterochromatin domain (reviewed in [24]). In the context of DNA repair, a phase-separated environment might selectively retain or exclude repair proteins to influence repair pathway choice and repair progression (Fig. 3). For example, efficient damage processing might rely on high retention of resection components inside the heterochromatin domain, or exclusion of NHEJ proteins from the domain. Accordingly, the early NHEJ component Ku80-GFP is mostly excluded from the HP1a domain in *Drosophila* cells [26], where repair largely occurs by HR [26,28,38]. Additionally, the heterochromatin domain might retain Smc5/6 and other early repair proteins (*e.g.*, dPIAS, Arp2/3, myosins) [26,28,39], while excluding later repair components (*e.g.*, Rad51, Rad54) [26,38]. In agreement, HP1a loss affects both phase separation and Rad51 exclusion from the domain. Additionally, local HP1a loss at repair sites during a normal repair cycle [26] might enable Rad51 recruitment and repair progression at the nuclear periphery.

A phase separated environment would also facilitate focus clustering inside the heterochromatin domain, promoting early damage processing. Consistent with this idea, repair focus clustering in heterochromatin does not depend on Arp2/3 [39], and relocalization of repair sites to outside the domain is frequently concurrent with the splitting of these clusters into smaller foci [26]. Further, exclusion of Arp2/3 activators (i.e., Scar and Wash) might promote filament formation only after repair sites have reached the heterochromatin domain periphery, explaining why filaments mostly form outside the heterochromatin domain.

Phase separation also enables fast regulated changes in the biophysical properties of the domain, which could in turn facilitate repair progression. For example, chromatin modifiers or phosphorylation of heterochromatin components might change the biophysical properties of the heterochromatin domain to promote expansion and facilitate dynamic movements in response to DNA damage.

Intriguingly, other nuclear compartments required for heterochromatin repair are phase separated, including repair foci and nuclear pores. At repair sites, the early recruitment of poly(ADP-Ribose) polymerase 1 (PARP1) promotes poly-ADP-ribosylation, which results in recruitment of intrinsically disordered proteins (IDPs) and phase separation by liquid demixing [15]. While we do not know how these responses operate in heterochromatin, similar biophysical changes might promote the initial exclusion of repair foci from the heterochromatin domain, and their accumulation at the heterochromatin domain periphery where they interact with actin filaments. In agreement with this idea, the initial phase of focus relocalization in *Drosophila* cells is rarely concurrent with directed motions (Fig. 2D,E and [39]) or visible nuclear actin filaments [39,148], suggesting that independent separating forces contribute to these dynamics.

At nuclear pores, intrinsically disordered phenylalanine-glycine-rich nucleoporins (FG-Nups) generate a phase separated domain that forms a selective permeability barrier [16]. Recent studies further propose that FG-porins organize distinct territories within the pore, maintained by different types of FG motifs [134]. It is tempting to speculate that repair restart at the nuclear pores is influenced by this local environment, which might retain high concentration of components for strand invasion and HR progression.

Finally, F-actin and myosin-driven forces might be particularly critical to enabling the formation of protrusions of heterochromatin from the domain and relocalization of repair foci, counteracting surface tension of the phase separated HP1a domain. Thus, phase separation likely influences several aspects of DSB repair in heterochromatin, and understanding how pre-existing biophysical properties and damage-induced changes in these domains contribute to the spatial and temporal regulation of HR repair is an exciting challenge for future studies.

7. Conclusions and perspectives

Several studies in the past few years have shed light on a number of components that regulate heterochromatin repair in space and time to prevent aberrant recombination and enable 'safe' repair. Repair starts inside the heterochromatin domain, and continues outside with Rad51 recruitment. Nuclear F-actin and myosins generate pulling forces for relocalization, revealing a tight coordination between nuclear architecture, nuclear dynamics, and repair progression. These studies have

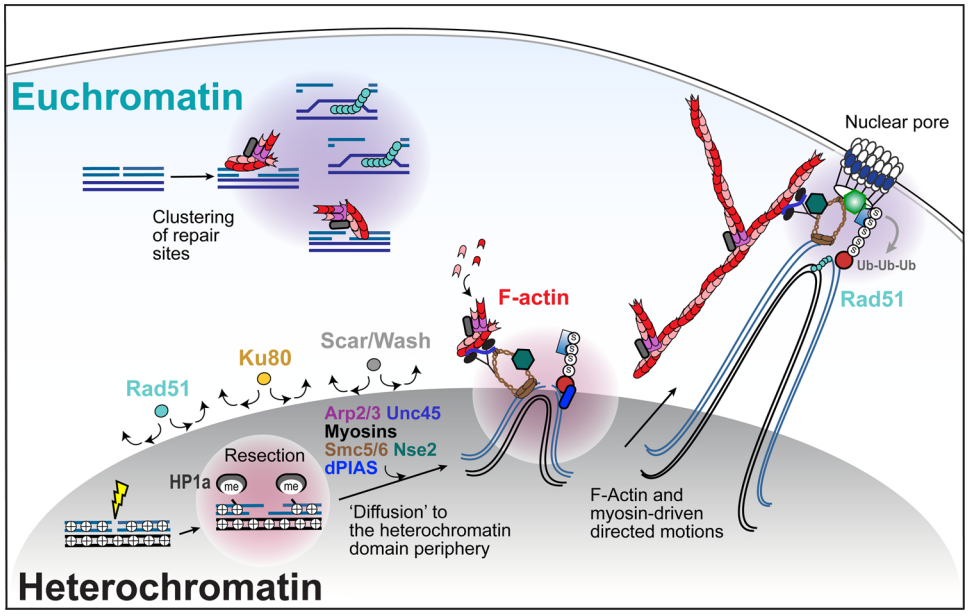

Fig. 3. Model for how phase separation might contribute to heterochromatin repair through selective protein accessibility. Liquid-liquid phase separation (LLPS) of heterochromatin, repair sites, and nuclear pores, might contribute to regulating heterochromatin repair in space and time. The heterochromatin domain might be permeable to resection and checkpoint components, while excluding NHEJ proteins (Ku80). The strand invasion component Rad51, and Arp2/3 activators (Scar, Wash), might also be excluded thus enabling resection and Arp2/3 recruitment inside the heterochromatin domain, but filament formation only at the heterochromatin domain periphery. Heterochromatin expansion might reflect global changes in the biophysical properties of the domain facilitating relocalization. The nuclear pore might provide a favorable environment for ubiquitination of SUMOylated components and for Rad51 recruitment. Finally, phase separation of heterochromatic repair sites might facilitate their diffusion from the core of the heterochromatin domain to its periphery, while in euchromatin it might promote clustering, resection and HR progression.

raised many new and exciting questions. How F-actin and myosins are regulated for heterochromatin repair is largely unclear. Targets of SUMOylation and checkpoint kinases in this context remain uncharacterized. How F-actin is disassembled during focus relocalization and the significance of this to repair is also unknown. The mechanisms restarting HR at the nuclear periphery and the role of ubiquitination in this step remain to be defined. The function of local and global chromatin changes in heterochromatin repair still needs to be understood, and the epigenetic targets of this regulation have just started to emerge. Importantly, understanding how the biophysical properties of heterochromatin as a phase separated environment contribute to different repair steps is an exciting direction for further investigation. Additionally, chromatin movement across nuclear domains is not uncommon and an important challenge is to establish the relevance of transient nuclear filaments and motors in nuclear dynamics for different functions, including transcription, chromosome territory repositioning, and DNA replication. Heterochromatin silencing [135,136], HR repair [137–140], nuclear periphery [141], and actin/myosin components [142] deteriorate with age, suggesting these declines as a contributor to repair defects and genome instability observed in older organisms [143–146] (reviewed in [33,46]). Thus, understanding heterochromatin repair mechanisms is expected to open new opportunities for addressing human disease, and the tools are now in place for exciting new discoveries in the upcoming years.

Declaration of Competing Interest

The authors declare no conflicts of interests.

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