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SHORT REPORT

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Cervantes and Quijote protect heterochromatin from aberrant recombination and lead the way to the nuclear periphery

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ABSTRACT

Repairing double-strand breaks (DSBs) is particularly challenging in heterochromatin, where the abundance of repeated sequences exacerbates the risk of ectopic recombination and chromosome rearrangements. In *Drosophila* cells, faithful homologous recombination (HR) repair of heterochromatic DSBs relies on a specialized pathway that relocalizes repair sites to the nuclear periphery before Rad51 recruitment. Here we show that HR progression is initially blocked inside the heterochromatin domain by SUMOylation and the coordinated activity of two distinct Nse2 SUMO E3 ligases: Quijote (Qjt) and Cervantes (Cerv). In addition, the SUMO-targeted ubiquitin ligase (STUbL) Dgrn, but not its partner dRad60, is recruited to heterochromatic DSBs at early stages of repair and mediates relocalization. However, Dgrn is not required to prevent Rad51 recruitment inside the heterochromatin domain, suggesting that the block to HR progression inside the domain and relocalization to the nuclear periphery are genetically separable pathways. Further, SUMOylation defects affect relocalization without blocking heterochromatin expansion, revealing that expansion is not required for relocalization. Finally, nuclear pores and inner nuclear membrane proteins (INMPs) anchor STUbL/RENi components and repair sites to the nuclear periphery, where repair continues. Together, these studies reveal a critical role of SUMOylation and nuclear architecture in the spatial and temporal regulation of heterochromatin repair and the protection of genome integrity.

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Introduction

Heterochromatin accounts for roughly 30% of fly and human genomes and mostly comprises repeated DNA sequences organized around the centromeres.[1-4] About half of these sequences consist of simple 'satellite' repeats, predominantly tandem 5-base pair sequences repeated for hundreds of kilobases to megabases, while the rest is composed of scrambled clusters of transposable elements and about 250 isolated genes.[2,4] Heterochromatin is mostly transcriptionally silent and it is likely maintained in multicellular eukaryotes because of its role in centromere establishment,[5] but the abundance of repeated sequences in these regions poses unique challenges to maintaining genome integrity.[6,7] Repeated sequences associated with different chromosomes can engage in ectopic recombination during DSB repair, leading to chromosome rearrangements and widespread genome instability.[6,7] HR starts when DSBs are resected to form single-stranded DNA (ssDNA) filaments, which invade 'donor' homologous sequences used as templates for DNA synthesis and repair.[8] In single copy sequences, a unique donor is present on the sister chromatid or the homologous chromosome, and repair is largely 'error free'.[8] In heterochromatin, however, the availability of thousands to millions of potential donor sequences can initiate unequal sister chromatid exchanges or inter-chromosomal recombination, leading to deletions, duplications, translocations, and formation of dicentric or acentric chromosomes.[6,7] Despite this danger, HR is extensively used to repair heterochromatic DSBs in both *Drosophila* and mammalian cells,[9-12] suggesting that specialized mechanisms regulate HR repair in heterochromatin while preventing aberrant recombination.

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Drosophila heterochromatin is organized in a distinct nuclear domain, providing unique advantages for imaging approaches.[10,13] Repair progression is also detectable cytologically, because most repair proteins form 'repair foci' when recruited to DSBs.[7] Using a combination of live imaging and fixed cell studies in *Drosophila* cells, we previously discovered that ionizing radiation (IR)-induced DSBs are quickly detected and processed inside the heterochromatin domain.[10] Next, the heterochromatin domain expands and repair sites display a striking relocalization to outside the domain.[10,11,14,15] Notably, a similar heterochromatin expansion and relocalization of repair sites occur in mouse cells, where heterochromatin is organized in several nuclear domains called 'chromocenters',[7,16-18] suggesting that repair mechanisms in heterochromatin are evolutionarily conserved. Furthermore, our studies in *Drosophila* cells revealed that repair progression is initially halted inside the heterochromatin domain[10,15] and resumes after the relocalization of repair sites to nuclear pores and INMPs at the nuclear periphery.[15] Disruption of this pathway results in chromosome rearrangements and widespread genome instability, revealing its importance for genome integrity.[10,15] We proposed that relocalization promotes 'safe' HR while preventing aberrant recombination by isolating DSBs and their 'proper' templates (homologous chromosomes or sister chromatids) away from identical but ectopic sequences on non-homologous chromosomes, before strand invasion.[7,10,15]

Here we will summarize our current understanding of this pathway, with a particular emphasis on early repair steps. We will highlight some of the most important open questions in the field and present new evidence that SUMOylation plays a key role in blocking HR progression inside the heterochromatin domain. We also identified the E3-SUMO ligase Cerv as a new component required to protect heterochromatin from aberrant recombination. Further, we show that SUMO-E3 ligases are not required for heterochromatin expansion, revealing that expansion of the domain is not sufficient to induce relocalization of repair sites. Finally, the STUbL protein Dgrn is recruited to DSBs inside the heterochromatin domain, potentially contributing to the targeting of DSBs to the nuclear periphery. Together, these studies unmask novel roles for SUMOylation and the nuclear periphery in the stability of repeated sequences and genome integrity.

Homologous recombination repair efficiently starts inside the heterochromatin domain, but Rad51 recruitment is temporarily blocked

Heterochromatin had long been considered less accessible than euchromatin to repair machineries, because of its compaction and silent state. However, γH2Av and Mu2/Mdc1 foci, which mark DSB detection and signaling, form inside the heterochromatin domain with kinetics similar to those in euchromatin.[10] Even more surprisingly, proteins recruited to DSBs after resection, such as ATRIP and TopBP1,[19,20] form foci in heterochromatin faster than in euchromatin.[10] These observations suggest that not only is heterochromatin not a barrier to DSB detection and signaling, but certain repair steps are even more efficient in heterochromatin.

Why heterochromatin is particularly responsive to damage is still unclear, but studies in euchromatin suggest that the abundance of silencing components and the more compact state of heterochromatin might play a role in this response. In fact, in mammalian cells the transient recruitment of HP1 proteins to euchromatic DSBs promotes damage signaling and resection,[21-27] while artificial tethering of HP1 to undamaged euchromatic sites triggers chromatin compaction and a damage response.[28] Thus, the typical concentration of 'silent' epigenetic marks in heterochromatin and/or damage-induced modifications of these components might facilitate early damage processing inside the heterochromatin domain.[10,16,29,30] Understanding which repair steps are particularly efficient inside the domain and the molecular mechanisms promoting these responses are important goals for future studies.

While early steps of HR proceed inside the heterochromatin domain, Rad51 recruitment to foci does not occur until repair sites have left the domain.[10,15] This block relies on HP1a *via* recruitment of the Smc5/6 complex,[10] suggesting a 2-way function for HP1 proteins: promoting DSB signaling and resection while blocking later repair steps (Rad51 recruitment and strand invasion). In agreement with this model, HP1a is locally removed or 'loosened' during Rad51 recruitment at later steps of repair.[10] What regulates this response in *Drosophila* is still unknown, but studies in mammalian cells suggest that post translational modifications of HP1

proteins, HP1 interacting factors, or chromatin modifiers might contribute to HP1 displacement from the chromatin during heterochromatin repair.[16,23,29,30]

SUMOylation by Nse2/Quijote blocks HR progression inside the heterochromatin domain

Observing that Rad51 foci do not form inside the heterochromatin domain[10,15] raises the question of what molecular mechanism blocks HR progression in heterochromatin in addition to, or downstream from, Smc5/6. Among the first proteins identified for this role are two SUMO E3 ligases: dPIAS and the Smc5/6 subunit Nse2/Qjt.[10,15] These components are recruited to heterochromatic repair sites before relocalization, and are independently required to block Rad51 recruitment.[15] Nse2/Qjt seems to play a primary role in this function, given that Nse2/Qjt RNAi results in a stronger effect than dPIAS RNAi, *i.e.* higher number of Rad51 foci inside the heterochromatin domain in response to IR.[15]

Abnormal formation of Rad51 foci inside the heterochromatin domain is also observed after SUMO RNAi, supporting the hypothesis that SUMOylation is the critical function required for blocking Rad51 recruitment.[15] However, SUMO RNAi might have pleiotropic effects in cells, and a direct assessment of the role of SUMOylation was lacking. We addressed this by generating Nse2/Qjt mutations in the SP-RING domain (Qjt-SA) that specifically abolishes the SUMO-E3 ligase activity[31] (Fig. 1A, B). We created stable lines expressing GFP-tagged versions of Qjt-WT (wild type) or Qjt-SA under the control of the endogenous Qjt promoter, and in which codon-swapping (cs) of the N-terminal region generates RNAi-resistant forms of these proteins (Fig. 1B). siRNAs directed against the N-terminal region efficiently deplete endogenous Nse2/Qjt mRNA (Fig. 1C), without affecting csQjt-WT and csQjt-SA proteins and their damage responses (Fig. 1D). Specifically, the association of csQjt (WT or SA) proteins to the heterochromatin domain before IR, or repair focus formation after IR, resembles what was previously observed for other Smc5/6 subunits, including Nse2/Qjt[10,15] (Fig. 1D). These observations suggest that Qjt-dependent SUMOylation is not required for Qjt recruitment to the heterochromatin domain or to repair foci, and validate the use of cells expressing csQjt-WT and csQjt-SA to address the role of SUMOylation in heterochromatin repair.

As previously shown, Qjt/Nse2 RNAi leads to the abnormal formation of Rad51 foci inside the heterochromatin domain at 60 min after IR, without affecting the total number of Rad51 foci (Fig. 1E and[15]). Expression of csQjt-WT fully rescues this phenotype, reducing the number of Rad51 foci in heterochromatin to the level observed in control RNAi cells (Fig. 1E). Conversely, expression of the SUMOylation-defective csQjt-SA does not reverse the high number of Rad51 foci in heterochromatin observed in the absence of endogenous Qjt (Fig. 1E). Expression of Qjt-SA in the absence of endogenous Qjt also results in the formation of heterochromatic DNA filaments that connect dividing cells, reflecting ectopic recombination (Fig. 1F). This phenotype resembles what we previously observed after RNAi of Smc5/6 or Nse2/Qjt.[10,15] We conclude that Qjt-dependent SUMOylation is necessary to prevent Rad51 recruitment inside the heterochromatin domain and aberrant recombination between repeated sequences.

Nse2 homologs Cervantes and Quijote independently block HR progression and aberrant recombination in heterochromatin

The gene *qjt* originated from retroposition of the parental gene *cerv*,[32] and the protein product Cerv shares 68% identity and 80% similarity with Qjt. The two genes are fast evolving,[32] but regions of high sequence similarity include both the SP-RING and the Smc5-binding domains (Fig. 1A). These observations suggest Cerv as a second homolog of Nse2 in *Drosophila*, prompting us to investigate whether Cerv also contributes to blocking HR progression in heterochromatin. Notably, Cerv RNAi depletion does not affect Nse2/Qjt recruitment to repair foci (Fig. 2A,B). Given that Nse2/Qjt recruitment to DSBs depends on Smc5/6,[15] this indicates that both Nse2/Qjt and Smc5/6 are normally recruited to DSBs in the absence of Cerv. However, similar to Qjt RNAi, Cerv RNAi depletion results in abnormal formation of Rad51 foci inside the heterochromatin domain at 60 min after IR, without affecting the total number of Rad51 foci (Fig. 2C). Simultaneous depletion of Cerv+Qjt results in additive effects, with higher numbers of Rad51 foci in the heterochromatin domain compared to each individual RNAi, and the magnitude of this effect resembles that

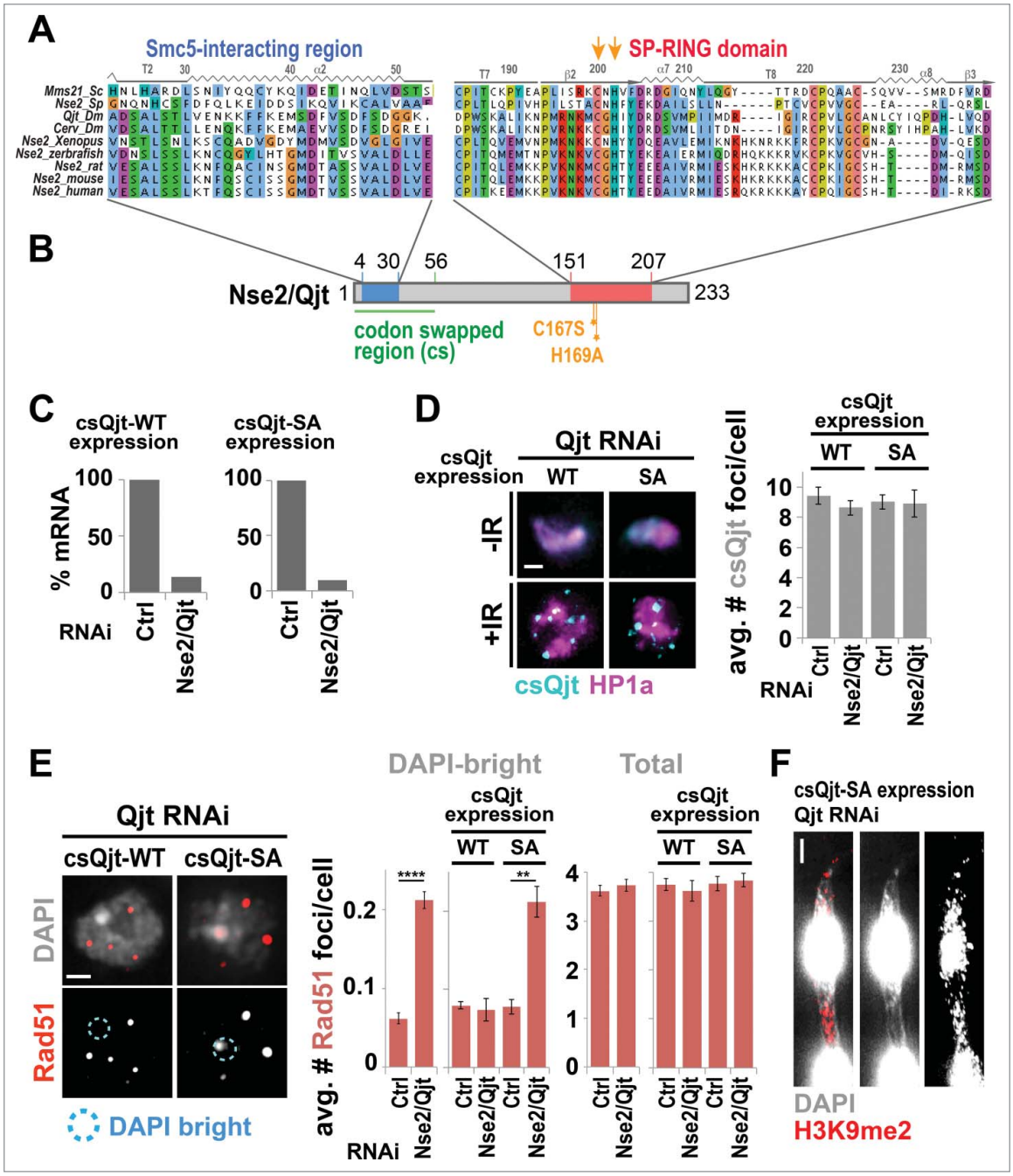

Figure 1. The SUMO E3-ligase activity of Quijote (Qjt/Nse2) is required to prevent abnormal Rad51 recruitment and aberrant recombination in heterochromatin. (A) Alignment of Nse2 homologs in the Smc5-interacting region (left) and the SP-RING domain (right). Arrows point to C and H residues that are essential for the SUMO E3-ligase activity. (B) Schematic representation of Nse2/Qjt, including the position of the codon-swapped region in csQjt (WT or SA) constructs. The Qjt-SA mutant sequence carries the indicated C167S and H169A mutations. (C) qPCR analysis of endogenous Nse2/Qjt mRNA in cells expressing GFP-csQjt (WT or SA mutant) shows the efficiency of depletion of endogenous Nse2/Qjt by siRNAs targeting the N-terminal region, relative to control RNAi. (D) Images (left) and quantification (right) of cells expressing GFP-csQjt (WT of SA) show that RNAi depletion of endogenous Nse2/Qjt does not affect either the enrichment in heterochromatin before IR (-IR) or focus formation at 10 min after IR (+IR) of csQjt/Nse2 (WT or SA). (E) Immunofluorescence (IF) analysis and quantification of cells expressing GFP-csQjt (WT or SA) after RNAi depletion of endogenous Nse2/Qjt, and fixed 60 min after IR, show that expression of the csQjt-SA mutant results in abnormal formation of Rad51 foci in DAPI-bright heterochromatin,[10,15] relative to csQjt-WT expression or control RNAi ($^{**}P < 0.01$, **** $P < 0.0001$, two-tailed Mann-Whitney test; n > 270 cells/RNAi condition). (F) Filament assay of cells expressing csQjt-SA, stained with DAPI and H3K9me2 after endogenous Qjt RNAi depletion, shows the abnormal formation of heterochromatic DNA filaments connecting dividing cells. Images are maximum intensity projections of all Z stacks in (D,F) or Z-stacks spanning the DAPI-bright region in (E). Scale bars = 1 μm. Error bars represent s.e.m. in (D), or s.d. across three independent experiments in (E).

of Smc5/6 RNAi (Fig. 2C). In addition, similar to Qjt RNAi, Cerv RNAi results in abnormal formation of heterochromatic DNA filaments between dividing cells (Fig. 2D), reflecting aberrant recombination between repeated sequences.[10,15] We conclude that the block to Rad51 recruitment inside the heterochromatin domain and the suppression of aberrant recombination among heterochromatic sequences requires the partially independent SUMO E3 ligases Cerv and Qjt, each of which might act as Smc5/6 complex subunit. These results also identify Cerv as a second *bona fide* Nse2 homolog in *Drosophila*. To our knowledge, this is the first documented case of two Nse2 paralogs working together in the same cell or organism. This discovery opens several questions about these paralogs, including differences and similarities in their recruitment mechanisms, SUMOylated targets, and possible 'divisions of labor' between Qjt and Cerv in cells and organisms.

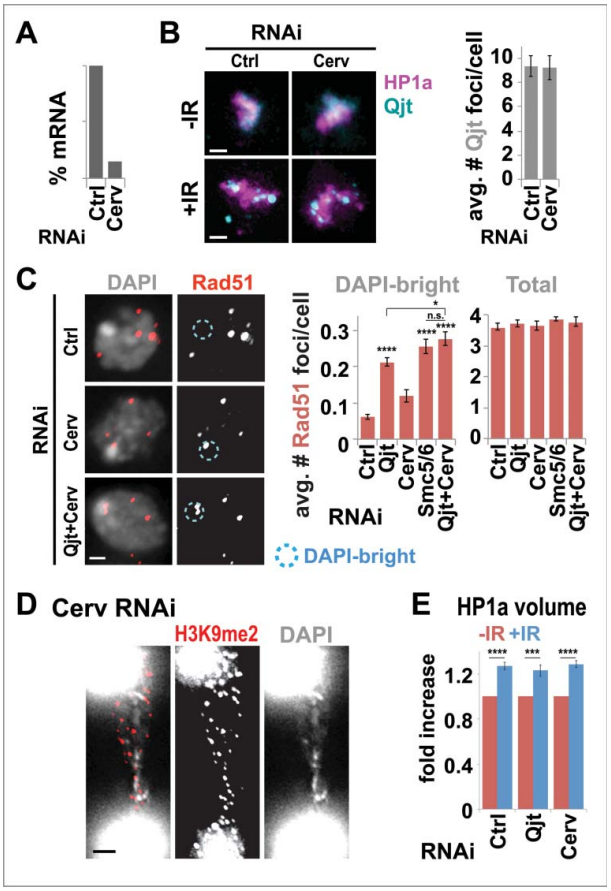

Figure 2. Cervantes (Cerv/Nse2) is required for blocking Rad51 loading in heterochromatin and preventing aberrant recombination. (A) qPCR analysis shows the efficiency of Cerv RNAi depletion. (B) Images (left) and quantification (right) of GFP-Nse2/Qjt signals show that RNAi depletion of Nse2/Cerv does not affect Nse2/Qjt enrichment in the HP1a domain before IR and its recruitment to repair foci after IR. (C) IF analysis (top) and quantification (bottom) of cells fixed 60 min after IR show the formation of Rad51 foci in DAPI-bright after RNAi depletion of Qjt or Cerv compared to Ctrl RNAi. Qjt+Cerv RNAi results in an additive effect ($^{*}P< 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, two-tailed Mann–Whitney test; n > 350 cells/RNAi condition). (D) As described in Fig. 1 F, except Kc cells were used and the assay was performed after Cerv RNAi. (E) Quantification of the fold increase in mCherry-HP1a volume after IR shows that RNAi depletion of Qjt or Cerv does not affect heterochromatin expansion, relative to Ctrl RNAi ($^{****}P < 0.0001$; $^{***}P < 0.001$, two-tailed Mann-Whitney test; n > 16 cells/RNAi condition). Images are maximum intensity projections of all Z stacks in (B,D) or Z-stacks spanning the DAPI-bright region in (C). Scale bars = 1 μm. Error bars represent s.e.m. in (B,E), or s.d. across three independent experiments in (C).

Heterochromatin expansion is not sufficient to relocalize heterochromatic DSBs

The function of heterochromatin expansion in DSB repair is still mysterious. Expansion is detected as an increase in the volume occupied by the HP1 domain in response to DSBs, in both mouse and *Drosophila* cells.[10,16] In *Drosophila*, this response depends on DNA damage checkpoint kinases (*i.e.*, ATM and ATR), and on resection components, suggesting a role of expansion in early steps of HR repair.[10] RNAi depletion of these components also affects the relocalization of repair sites suggesting that expansion might be responsible for relocalization. Accordingly, the peak of expansion corresponds to the time when DSBs move to outside the heterochromatin domain.[10] Because Smc5/6 and SUMO E3 ligases are required for relocalizing heterochromatic DSBs,[10] we tested the hypothesis that Cerv and Qjt also mediate heterochromatin expansion. However, RNAi depletion of Cerv or Qjt does not result in defective expansion of the HP1a domain in response to IR (Fig. 2E). In addition to providing a better understanding of the specific roles of Cerv and Qjt in heterochromatin repair, this observation reveals that expansion and relocalization are genetically separate pathways. Checkpoint and resection components are required for both heterochromatin expansion and relocalization of DSBs. Conversely, SUMO-E3 ligases mediate DSB relocalization but not heterochromatin expansion. Given that Qjt RNAi affects relocalization without blocking expansion, we conclude that expansion is not sufficient to induce the relocalization of heterochromatic DSBs. However, whether expansion facilitates relocalization, is still unclear.

The STUbL Dgrn associates with DSBs inside the heterochromatin domain and mediates DSB relocalization

What is the signal responsible for targeting heterochromatic DSBs to the nuclear periphery? Our studies so far suggest that SUMOylation might also mediate this function, because SUMO-E3 ligases are not only necessary to block HR progression inside the heterochromatin domain, but are also required for relocalization of heterochromatic repair sites.[15] Corroborating this hypothesis, studies in yeast G1 cells revealed that artificial tethering of polySUMO tails to a chromatin site triggers its targeting to nuclear pores, even in the absence of DNA damage.[33] Interestingly, the SUMO-binding protein Dgrn also mediates relocalization of heterochromatic DSBs in *Drosophila* cells.[15] However, contrary to SUMO and SUMO-E3 ligases, Dgrn is not required to block HR progression inside the heterochromatin domain, revealing that DSB relocalization and the suppression of HR progression are genetically separate pathways.[15] SUMOylation regulates both, but STUbL/Dgrn is only required for relocalization.

How Dgrn mediates DSB relocalization is still unclear. Dgrn is mostly concentrated at the nuclear periphery,[15] where it may act after relocalization by anchoring repair sites and potentially restarting repair (see following section). However, this protein is also frequently found in the nucleoplasm of yeast, human, and *Drosophila* cells,[15,33-35] and might potentially be recruited to DSBs inside the heterochromatin domain to initiate relocalization. We tested this directly by expressing tagged versions of Dgrn, and quantifying its colocalization with γH2Av foci at different time points after IR. As in most cell types,[36] the total number of γH2Av foci in *Drosophila* cells peaks at 30 min after IR followed by a slow decline during repair (Fig. 3A and [10], total, γH2Av). However, repair foci inside the heterochromatin domain are characterized by a sharp peak at 10 min after IR, followed by a drastic drop during relocalization (Fig. 3A and [10], DAPI-bright, γH2Av). Dgrn is efficiently recruited to γH2Av foci in DAPI-bright at 10 min after IR, similar to dPIAS (Fig. 3A, DAPI-bright, and[15]). This places both Dgrn and dPIAS at heterochromatic repair sites before relocalization. Interestingly, STUbL proteins typically associate with polySUMOylated targets,[37] suggesting the possibility that dPIAS-dependent polySUMOylation of unknown components triggers Dgrn recruitment to DSBs at early steps of repair. Notably, studies in yeast revealed that artificial tethering to chromatin of the Dgrn homolog Slx5 is sufficient to target a genomic site to nuclear pores in G1 cells.[33] Whether this is a universal property of STUbL proteins and whether this activity might function outside G1 phase is unknown, but it is tempting to speculate that the recruitment of Dgrn to heterochromatic DSBs before relocalization reflects an early role for this component in targeting repair sites to the nuclear periphery. In this model, poly-SUMOylation would regulate both HR progression (by blocking Rad51 recruitment), and relocalization (by recruiting Dgrn), thus ensuring tight coordination between the two processes.

Heterochromatic repair sites associate with the nuclear periphery via STUbL/dRad60 to continue HR repair in a 'safe' environment

After relocalization to the nuclear periphery, repair sites colocalize with both the nuclear pore Nup107-160 complex and INMPs of the Mps3 family, Koi and Spag4.[15] Nuclear pore proteins and INMPs are also present in the nucleoplasm, where they regulate transcription and possibly chromatin dynamics.[38-41] However, Nup107-160, Koi and Spag4 do not appear enriched at repair foci in the nucleoplasm, and their RNAi depletion results in repair sites failing to associate with the periphery and exploring more of the nuclear space.[15] These observations reveal that nuclear pores and INMPs perform 'anchoring' functions for repair sites after relocalization, rather than being directly involved in relocalization *per se*.

What is the role of the nuclear periphery in heterochromatin repair? Our studies suggest that the association of repair sites with the nuclear periphery is necessary for restarting HR repair. In fact, ATRIP focus intensity drops after relocalization of repair sites to the nuclear periphery indicating HR progression,[10,42] while components required for strand invasion (Brca2 and Rad51) are recruited to these sites.[15] Further, the reduction of ATRIP focus intensity is not observed in the absence of nuclear periphery anchoring components,[15] revealing that anchoring is not only concomitant with, but also required for repair progression. Notably, whether repair is also completed at the nuclear periphery or repair sites are released to

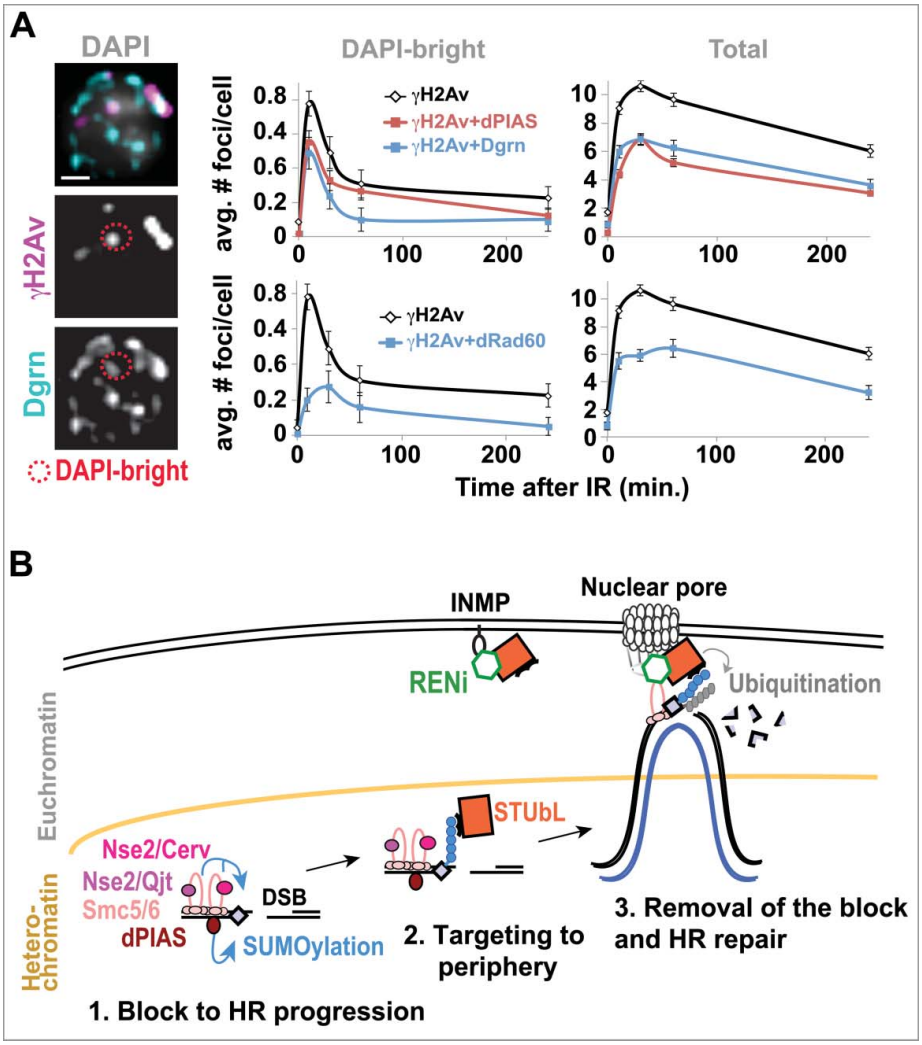

Figure 3. Dgrn is recruited to heterochromatic DSBs before relocalization. (A) IF analysis (left) at 10 min after IR and quantification (right) at indicated time points after IR show the number of GFP-Dgrn, FHA-dRad60, or dPIAS foci colocalizing with γH2Av foci in DAPI-bright regions or in the whole nucleus (Total). The number of Dgrn foci associated with γH2Av foci in DAPI-bright at 10 min is significantly higher than that observed at 60 min ($p < 0.05$, two-tailed Mann–Whitney test). Data for dPIAS are from.[15] Images show one Z-stacks spanning the DAPI-bright region. Scale bar = 1 μm. Error bars represent s.e.m. (B) Model for the molecular mechanism of heterochromatic DSB repair. DSBs inside the heterochromatin domain are quickly resected and recruit dPIAS, Nse2/Cerv and Nse2/Qjt SUMO-E3 ligases to SUMOylate repair targets and prevent Rad51 recruitment. PolySUMOylation recruits the STUbL Dgrn, leading to DSB relocalization to nuclear pores and INMPs. At the nuclear periphery, STUbL/RENi-dependent ubiquitination of poly-SUMOylated targets removes the block to HR progression to enable Rad51 recruitment, strand invasion, and the progression of repair. We propose that sister chromatids and/or homologous chromosomes (blue lines) also relocalize in concert with the lesion, providing homologous templates for 'safe' heterochromatin repair away from ectopic sequences.

complete repair elsewhere is still unclear. Nevertheless, the loss of nuclear periphery components (*i.e.*, Nup107, Koi and Spag4) leads to defective IR sensitivity, persistent 'unrepaired' heterochromatic DSBs, and formation of micronuclei containing heterochromatin marks that likely result from the loss of large chromosome fragments in mitosis.[15] Furthermore, fly mutants in these components display a dramatic increase in chromosome aberrations in larval tissues, mostly characterized by chromosome fusions at centromeric regions, loss of entire chromosome arms, and extra copies of repeated sequences.[15] These phenotypes typically result from lack of repair or incorrect repair of DSBs in pericentromeric regions,[15] revealing the importance of the nuclear periphery and the relocalization pathway for the completion of heterochromatin repair.

The nature of the signal that promotes repair continuation at the nuclear periphery is also unknown, but our studies suggest the STUbL protein Dgrn and its partner the RENi protein dRad60 as potential regulators of this function. Dgrn and dRad60 are highly

enriched at both nuclear pores and INMPs,[15] and their physical interaction with Smc5/6 in response to IR may contribute to docking repair sites to the nuclear periphery.[15] In addition, STUbLs typically ubiquitinate polySUMOylated proteins to target them for proteasome-mediated degradation[43-46] or to promote their interaction with other proteins.[47] Thus, Dgrn activation at the nuclear periphery might provide the molecular 'switch' necessary to remove the polySUMOylated block to HR progression and continue repair.

The role of dRad60 in this pathway is still enigmatic. Contrary to Dgrn, dRad60 is not recruited to γH2Av foci inside the heterochromatin domain (Fig. 3A), suggesting that this protein functions mostly at the nuclear periphery and not in relocalization *per se*. In agreement, artificial tethering of the dRad60 homolog Esc2 to a chromatin site in yeast does not trigger its targeting to the nuclear periphery.[33] Thus we suggest that dRad60 might act as a regulator of STUbL function on its targets, similar to the role suggested for the budding and fission yeast homologs Esc2 and Rad60, respectively.[48-50] In this model, Dgrn would interact with polySUMOylated targets inside the heterochromatin domain to mediate relocalization, and its ubiquitinating activity would become active upon association with dRad60 at the nuclear periphery. Understanding these regulations and the interplay between Dgrn-dRad60 and other repair and nuclear periphery components remain important open questions in the field.

Together, these studies reveal a key role of the nuclear periphery in completing HR repair of damaged heterochromatic repeated sequences, and suggest the compartmentalization of STUbL/RENi proteins in this location as a key element required to restart repair in a 'safe' environment.

Concluding remarks

Given the abundance of pericentromeric repeated sequences in multicellular eukaryotes, and the danger of aberrant recombination in this domain, it is not surprising that HR repair of those sequences requires a highly complex mechanism. Spatial and temporal separation of repair steps seems to be the key for faithful completion of HR repair in heterochromatin while suppressing ectopic recombination. Our studies so far have identified several repair and nuclear architecture components required to orchestrate this process. Based on these studies, we propose (Fig. 3B) that the early activation of resection inside the heterochromatin domain promotes the relaxation of the heterochromatin domain and channels repair progression into the tightly regulated HR pathway,[51] while SUMOylation of repair targets by dPIAS, Nse2/Qjt and Nse2/Cerv prevents Rad51 recruitment and strand invasion of ectopic sequences. PolySUMOylation of these targets might also recruit STUbL/Dgrn to repair sites, promoting their relocalization to the nuclear periphery. After relocalization, removal of this block to HR progression by the STUbL-RENi complex Dgrn-dRad60 promotes Rad51 recruitment and strand invasion away from the bulk of ectopic repeated sequences present in the heterochromatin domain. This model also predicts that homologous templates are available to complete repair at the nuclear periphery. Cohesion among the sister chromatids and, in *Drosophila*, mitotic pairing among homologous chromosomes,[52] might be sufficient to assure relocalization of homologous templates in concert with the broken chromosome. While several aspects of this model still await confirmation, these studies have revealed the importance of the nuclear architecture and the relocalization pathway in heterochromatin repair and genome stability.

Interestingly, several features of this pathway resemble a mechanism that targets persistent DSBs, telomeric lesions, and collapsed replication forks to the nuclear periphery in *S. cerevisiae*.[33,34,53-58] In this system, cell cycle phases and the type of lesion influence the final site for repair (nuclear pores vs INMPs),[33,56,57] and poly-vs mono-SUMOylation coordinate the targeting to one or the other location.[33] Whether similar regulations operate during heterochromatin repair is still a major unknown, and the relevant SUMOylation targets in heterochromatin are still mysterious. The similarity with yeast is particularly surprising, given that *S. cerevisiae* lacks the long pericentromeric repeats that characterize most multicellular eukaryotes, as well as H3K9 methylation and HP1 proteins that are necessary for the relocalization and protection of heterochromatic DSBs. We suggest that common signaling mechanisms trigger these responses in different contexts, such as prolonged or 'halted' resection and ssDNA-mediated signaling leading to polySUMOylation of repair targets.[59,60]

Importantly, most DSBs at the nuclear periphery in *Drosophila* cells are heterochromatic, suggesting the nuclear periphery as a specialized site for repairing DSBs that originated in the heterochromatin domain. As a result of these studies, relocalization of DSBs to the nuclear periphery is emerging as potentially one of the most important mechanisms safeguarding genome stability in multicellular eukaryotes. Thus, there is a pressing need to identify the molecular mechanisms involved, establish their conservation in human cells, and determine the contribution of their deregulation to human disease. The tools are now in place for exciting advancements of this field in the near future.

Methods

Plasmids

The GFP-csQjt-WT plasmid was generated by replacing the pCopia promoter of pCopia-GFP-Nse2[10] with the Nse2/Qjt promoter amplified from genomic DNA of Kc cells using the following oligos: 5′GACATGTAC ATTTAAAAACTTAAATAAAACGTTG and 5′ATC TGCAGTTTATCCAGAATATTTAAGC. The PCR product was cloned into pCopia-GFP-Nse2 after PciI/ PstI digestion. Qjt-SA mutants were generated using the QuikChange Site-Directed Mutagenesis (Agilent Technologies), and csQjt (WT or SA) plasmids were generated by substitution of the N-terminal region of Qjt with the following re-annealed oligos, after AscI/ NdeI digestion: 5′CATATGCACGACGTTGACAGC ATGTTCGACGGATTGTTCCACGGATTGTTCCAG AAGTTTCTTATCTTGCCACCATCGGAAAAGTCG CTGACAAAGTCGCTCATCTCTTTAAAGAATTTC TTATTCTCGACCAGGGTGCTCAAGGCGCTGTCG GCCAAATAATTAAACTCCATGG, and 5′GGCGCG CCATGGAGTTTAATTATTTGGCCGACAGCGCCT TGAGCACCCTGGTCGAGAATAGAAATTCTTTAA AGAGATGAGCGACTTTGTCAGCGACTTTTCCGA TGGTGGCAAGATTAAGAAACTTCTGGAACAATC CGTGGAACAATCCGTCGAACATGCTGTCAACGT CGTGCA.

dsRNA synthesis and sequences

dsRNAs were synthesized as described in.[15] Qjt dsRNA was prepared with the oligos: 5′CTAATAC GACTCACTATAGGGAGATGGAATTCAATTACCT TGCTGATTCCGC and 5′CTAATACGACTCACT ATAGGGAGGACTACATTCACGGCGTGCT. Cerv dsRNA was prepared with the oligos: 5′TAATACGA CTCACTATAGGGTGATCATCACGGACAACAT TG and 5′TAATACGACTCACTATAGGGTACATA CGTGCATACTGCACT.

Focus quantifications

Quantifications of repair foci in live and fixed samples were performed as previously described.[10,15] In Fig.1E, the quantification of Rad51 foci in cells expressing GFP-csQjt-WT or GFP-csQjt-SA was done only in cells expressing a GFP signal.

Quantification of heterochromatin expansion

For quantifying the HP1a expansion in time-lapse experiments, 3D volumes occupied by mCherry-HP1a were analyzed for consecutive time points in deconvolved time-lapse images of individual nuclei. The same field of cells was imaged before and every 10 min after IR, for 1h. Deconvolution was done with softWoRx (Applied Precision), and volumetric analysis with Imaris (Bitplane). The HP1a domain was identified using the 'surface creation' module in Imaris, and the same threshold was applied to images before and after IR. The fold increase in HP1a volume was calculated by dividing the max volume after IR to the volume before IR, for each cell.

qPCR

qPCR was performed as described in.[15] Primer sequences were:5′TCAGTCGGTTTATTCCAGTC and 5′CAGCA ACTTCTTCGTCACACA for Atc5C; 5′CACGTGGC ATTTGCTTAAAA and 5′GCTGAAATCGGACACGA AAT for Nse2/Qjt; 5′TTCGAGGAAGTCTGGAAGGA and 5′TTGGACCAGGGATCGTAGAG for Nse2/Cerv.

Sequence alignments

Alignment between Cerv, Qjt, and other Nse2 homologues was done using Clustal Omega and manually adjusted based on.[61] Calculation of identity and similarity between the two proteins was also done with Clustal Omega.

Other methods

Cell culturing, IR treatments, generation of stable cell lines, RNAi depletions, imaging, immunofluorescence,

and filament assays, were performed as described in [7,15]. The control (Ctrl) used for all RNAi experiments is RNAi depletion of the *brown* gene.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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