Double-strand breaks in facultative heterochromatin require specific movements and chromatin changes for efficient repair

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Abstract

DNA double-strand breaks (DSBs) must be properly repaired within diverse chromatin domains in order to maintain genome stability. Each chromatin domain has specific properties that can influence the DNA repair response. Whereas euchromatin has an open structure and is associated with active transcription, facultative heterochromatin is more compact and essential to silence developmental genes. Facultative heterochromatin is enriched for Histone H3 Lysine 27 tri-methylation (H3K27me3) and polycomb group proteins, and accumulates in sub-nuclear foci, called polycomb bodies. Whether the specific chromatin properties of facultative heterochromatin require distinct DSB repair dynamics remains unknown. Here we integrate inducible single DSB systems in eu- and facultative hetero-chromatin in *Drosophila* animals and find that facultative heterochromatic DSBs rapidly move outside polycomb bodies within minutes after their appearance. In line with these specific DSB dynamics, we find a local loss in the canonical heterochromatin marker H3K27me3 at break sites. We demonstrate that DSB movement and loss of H3K27me3 at heterochromatic DSBs both depend on the histone demethylase dUtx. Importantly, this dUtx-mediated loss of H3K27me3 is required for completion of homologous recombination at facultative heterochromatic DSBs. We conclude that DSBs in polycomb bodies are subjected to dUtx-mediated loss of the silencing mark H3K27me3, which promotes DSB movement and timely repair by homologous recombination. Thus, facultative heterochromatin requires distinct chromatin changes to promote safe DSB repair.

INTRODUCTION

Eukaryotic cells are continuously exposed to factors that can break or chemically alter their DNA. One particularly dangerous type of DNA damage is a DNA double-strand break (DSB), in which both strands of the DNA helix are severed. Improper repair of DSBs can directly lead to insertions, deletions and chromosomal rearrangements associated with disease development including cancer[1]. Fortunately, our cells have evolved mechanisms to repair these DSBs of which the two main pathways are Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR). During NHEJ, the broken DNA ends undergo limited end processing and are directly ligated, which can result in small insertions or deletions (indels) at the break site and is therefore often considered error-prone[2]. HR, on the other hand, is usually more error-free since it relies on a homologous template to repair the DSB. During the process of HR, the broken DNA ends undergo 5' to 3' end-resection, generating a 3' single-stranded DNA (ssDNA) overhang. This overhang invades a homologous sequence on the sister chromatid or the homologous chromosome, which serves as a repair template. The choice of DSB repair pathway depends on multiple aspects, including cell cycle phase, the sequence context of the surrounding DNA, as well as the pre-existing chromatin state[2–4].

The eukaryotic nucleus consists of a variety of chromatin domains each characterized by specific molecular and biophysical properties. Whereas euchromatin is generally associated with open chromatin and actively transcribed genes, heterochromatin is a more compact type of chromatin associated with transcriptional silencing. One specific type of heterochromatin is facultative heterochromatin, which is essential to silence specific developmental genes throughout organismal development. This type of chromatin can cover large genomic distances (e.g. developmental genes such as Hox genes)[5], or regulatory regions (e.g. promoters)[6]. Facultative heterochromatin is enriched for Histone H3 Lysine 27 trimethylation (H3K27me3) and polycomb group proteins (PcG), and accumulates in sub-nuclear foci, called polycomb bodies[7–9]. Polycomb bodies cluster PcG-bound transcriptionally repressed genomic regions to maintain correct silencing of developmental genes[9–13]. Although the DSB response in open, euchromatic regions has been extensively studied, the DSB repair response in facultative heterochromatin remains largely unknown. Understanding the mechanisms by which silenced developmental genes in facultative heterochromatin maintain their integrity in the presence of DNA damage can give insights into how misregulation of this response could lead to disease development in the long run.

In the past decade, it has become increasingly clear that the pre-existing chromatin state can directly influence the DSB repair response. For example, DSBs in actively transcribed regions are prone to clustering and repair by HR[14,15]. Moreover, DSBs in centromeres[16], nucleoli[17–19] and constitutive heterochromatin domains [16,20–22] have been found to move outside the respective domains to facilitate HR repair. These domain-specific DSB movements have been hypothesized to promote access of repair proteins to the DSB[17], or prevent aberrant recombination between repetitive sequences within constitutive heterochromatin[20]. Previous evidence also suggests that DSB movements can occur within the inactive X chromosome[23], which is a specific type of facultative heterochromatin enriched for both H3K27me3 as well as Histone H3 Lysine 9 tri- methylation (H3K9me3)[24]. Irradiation of female human fibroblasts resulted in the specific exclusion of DSB repair proteins outside the inactive X chromosome, suggestive of DSB movement[23]. However, live imaging to accurately follow DSB dynamics within facultative heterochromatin has never been performed. More importantly, how polycomb bodies *in vivo* respond to DSBs and whether this chromatin environment promotes specific DSB movements remains unknown.

Various histone modifications have been identified to play a role at DSBs in euchromatin[3,25]. Silencing histone modifications, including H3K27me3[26,27] and H3K9me2/3[28,29] are deposited at DSBs in euchromatin resulting in transient heterochromatinization and potential transcriptional silencing at the DSB site. To restart transcription after DSB repair in euchromatin, active removal of H3K27me3 by the mammalian histone demethylase UTX (Ubiquitously transcribed Tetratricopeptide repeat on X chromosome) has been suggested to occur specifically in cancer cells, not healthy fibroblasts[30]. In contrast to the accumulation of silencing marks at euchromatic DSBs, we previously identified a loss of H3K9me2/3

at DSBs within *Drosophila* constitutive heterochromatin[31,32]. These findings suggest that eu- and heterochromatin regions require differential changes in silencing histone modifications to repair their DSBs. Whether specific H3K27-modifying activities are needed to repair DSBs in H3K27me3-enriched facultative heterochromatin domains remains untested.

To study the dynamic DSB response in facultative heterochromatin *in vivo*, we here integrate inducible single DSB systems[22,33] in eu- and facultative hetero-chromatin regions in *Drosophila* animals. Using high-resolution live imaging, we find that the majority of DSBs in polycomb bodies rapidly move outside these domains within ten minutes after their appearance. Moreover, we find that facultative heterochromatic DSBs specifically induce a local decrease in the canonical heterochromatin histone mark H3K27me3, which is dependent on the histone demethylase dUtx. Early steps of HR can occur efficiently within polycomb bodies and are independent of dUtx, while dUtx-mediated loss of H3K27me3 at DSB sites is required for DSB movement and completion of HR. Together, our results reveal that DSBs in facultative heterochromatin move outside the compacted polycomb bodies to promote timely repair by HR.

RESULTS

Development of a single double-strand break system in facultative heterochromatin

To study DSB repair in facultative heterochromatin in detail in animal tissue, and directly compare responses to euchromatin, we generated a set of inducible single DSB systems in *Drosophila*. We integrated our previously established *in vivo* DR-*white* reporters[22,33] into three facultative heterochromatin regions and two euchromatin regions (**Fig.1A, B**). The DR-*white* reporter contains two inactive copies of the *white* gene, in which the upstream *white* gene contains a recognition site for the rare-cutting I-SceI endonuclease, and the downstream i*white* sequence is truncated, lacking the 5' and 3' ends of the *white* gene. Upon expression of I-SceI, DSBs are induced in the upstream *white* gene. DSB repair pathway usage can subsequently be determined by sequencing the resulting repair products; HR with the downstream i*white* sequence will generate an intact upstream *white* gene, while NHEJ will generate small insertions and deletions (indels) at the I-SceI recognition site (**Fig.1A**). We confirmed integration of the DR-*white* constructs in the targeted eu- and facultative heterochromatin regions by PCR (**Fig.1B**). In addition, we performed ChIP-qPCR (Chromatin Immuno-Precipitation followed by quantitative PCR (qPCR)) for the canonical facultative heterochromatin histone modification H3K27me3. As expected, the three DR-*white* integrations in facultative heterochromatin were enriched for H3K27me3 when compared to the two euchromatic insertions (**Fig.1C**).

To allow timed DSB induction, we combined our DR-*white* systems with either a heat-shock-inducible I-SceI transgene (*hsp70*.I-SceI, **Fig.1D**) or an ecDHFR-I-SceI transgene, which depends on trimethoprim (ecDHFR-binding ligand) to stabilize the ecDHFR-I-SceI protein (**Fig.1E**)[22]. We first tested the efficiency and inducibility of our DR-*white* systems by performing ChIP-qPCR for phosphorylated H2Av on Serine 137 (γH2Av, γH2AX in mammals), one of the earliest chromatin markers of DSB induction[34]. Heat-shock inducible expression of I-SceI (**Fig.1D**) resulted in a local increase in γH2Av levels within six hours at both euchromatic and heterochromatic DR-*white* loci (**Fig.1F, G**). Moreover, we find the appearance of single γH2Av foci in nuclei of fixed imaginal discs by immunofluorescence six hours after heat-shock-inducible I-SceI expression in larvae containing either a eu- or hetero-chromatic DR-*white* insertion (16-18% of cells contained a single γH2Av focus compared to 3-5% in control cells) (**Fig.S1A**). These results suggest efficient DSB induction in both eu- and hetero-chromatic loci.

To directly determine which DSB repair pathways play a role in facultative heterochromatin, we performed Sanger sequencing followed by TIDE analyses[35] on the DR-*white* reporters upon I-SceI expression. Feeding trimethoprim throughout development (3-4 days) to DR-*white* larvae expressing ecDHFR-I-SceI (**Fig.1E**) results in the appearance of repair products in both eu- and hetero-chromatin (**Fig.1H**). Repair rates vary slightly between integration sites (55-70%), possibly reflecting differential

cutting efficiency or repair timing at the different sites (**Fig.1H**). However, no apparent differences in the number of identified repair products were identified between heterochromatin and euchromatin, indicating that both domains undergo efficient DSB induction and repair. More importantly, repair pathway analyses revealed that I-SceI induced DSBs in both eu- and facultative hetero-chromatin regions employ HR (17-37%) and NHEJ (67-83%) to a similar extent (**Fig.1I**). In line with this, expression of hsp.I-SceI also resulted in the appearance of DSB repair products (7-19%) and similar HR and NHEJ repair percentages in eu- and hetero-chromatin DR-*white* integrations (**Fig.S1B, C**). Our heat-shock inducible system resulted in an overall lower number of repair products when compared to ecDHFR-I-SceI, likely reflecting the shorter duration of I-SceI expression (24 hours in hsp.I-SceI compared to 3-4 days in ecDHFR-I-SceI system). To confirm the role of HR repair at facultative heterochromatic DSBs, we depleted the end-resection HR protein CtIP in 3rd instar larvae using RNAi (**Fig.S1D**) and analyzed DR-*white* repair products following hsp.I-SceI induction (**Fig.S1E, F**). As expected, loss of CtIP resulted in a decrease in HR repair products (3-11% compared to 17-33% in control) in both eu- and heterochromatin, indicating that the identified HR repair products by TIDE analyses indeed reflect end-resection dependent HR repair.

Together, these data reveal that both I-SceI systems (hsp.I-SceI/ecDHFR-I-SceI) efficiently induce DSBs with little variation between eu- and heterochromatin regions in the identified repair products, suggesting that both chromatin regions undergo similar DSB repair -efficiency and -pathway choice. In conclusion, we set-up an inducible single DSB system *in vivo* that allows us to perform detailed analyses of DSB repair in facultative heterochromatin, and directly compare it to the DSB response in euchromatin.

DSBs rapidly move outside polycomb bodies

Specific DSB spatiotemporal dynamics are associated with a variety of chromatin domains, such as centromeres[16], nucleoli[17–19] and constitutive heterochromatin[16,20,22]. These dynamics include the movement of DSBs to the periphery of the respective domain, as well as nuclear periphery[19,21]. Facultative heterochromatin forms distinct domains in the fly and mammalian nucleus, termed polycomb bodies[7–9]. These polycomb bodies serve to coalesce distant genomic regions into a stable, compact environment and thereby maintain proper silencing of developmental genes[9–13]. We wished to determine whether the distinct molecular- and biophysical- properties of polycomb bodies[36–38] could impact DSB dynamics and promote movements similar to those identified in other nuclear domains. To this end, we employed our DR-*white* systems to perform *in vivo* live imaging of single DSBs in facultative heterochromatin (**Fig.2A**) using fluorescently tagged Mu2 to visualize DSBs, and fluorescently tagged polyhomeotic-proximal (ph-p) to visualize polycomb bodies (**Fig.2B**). Mu2 is the *Drosophila* ortholog of mammalian MDC1, and directly binds γH2Av[39,40], while ph-p is one of the four core subunits of the *Drosophila* PRC1 (Polycomb Repressive Complex 1) and is enriched in fly polycomb bodies[41,42] (**Fig.2B**). Strikingly, our live-imaging analyses revealed that the majority of Mu2 foci (±70%) that appear within polycomb bodies move outside the domain within ten minutes (**Fig.2C, D, Fig.S2A**), while euchromatic DSBs that appear outside polycomb bodies remain outside until their disappearance (**Fig.S2B**). Importantly, these movements are not specific for I-SceI induced DSBs, since inducing DSBs in larval tissue using 5Gy gamma-radiation (IR) resulted in similar DSB dynamics in ph-p marked polycomb bodies. The majority of IR-induced Mu2 foci that appear within polycomb bodies move outside this domain within ten minutes after appearance (**Fig.2E, F**), whereas euchromatic DSBs are resolved outside polycomb bodies (**Fig.S2C**). Taken together, our live-imaging data demonstrate for the first time that facultative heterochromatic DSBs move outside polycomb bodies to continue repair.

Reduction in H3K27me3 at facultative heterochromatic DSBs is mediated by the histone demethylase dUtx

Considering the compact and silent state of facultative heterochromatin, we hypothesized that local chromatin changes could coincide with the specific DSB dynamics in polycomb bodies. To this end, we

assessed the levels of the canonical facultative heterochromatin histone modification H3K27me3 by ChIP-qPCR at DSB sites (**Fig.1F, 3A**). We observed a decrease in H3K27me3 (loss of 16-18%) at two of the three facultative heterochromatic DSB sites after I-SceI induction, while H3K27me3 at euchromatic DSB sites remained unchanged (**Fig.3A**). To exclude that the observed reduction in H3K27me3 is due to histone loss at the break site, we also performed ChIP-qPCR for histone H3, which did not reveal any significant differences in histone H3 levels at euchromatic and facultative heterochromatic DSB sites (**Fig.3B, Fig.S3A**). Since a reduction in H3K27me3 was only observed at two of the three heterochromatic sites, this suggests that not all heterochromatic DSBs induce loss of H3K27me3. Alternatively, our ChIP assay may not be sensitive enough to pick up on subtle differences in H3K27me3 levels. Nevertheless, these data reveal that DSBs in facultative heterochromatic DSB are accompanied by a local reduction in H3K27me3.

We hypothesized that the reduction in H3K27me3 levels at facultative heterochromatic DSBs could be mediated by a histone demethylase that actively removes the methyl groups from H3K27. In *Drosophila*, dUtx is the only protein described to be able to demethylate H3K27me3[43]. To determine whether dUtx removes the methyl group at facultative heterochromatic DSB, we depleted dUtx using RNAi in 3rd instar larvae (**Fig.3C**) and assessed H3K27me3 levels at the DSB site using ChIP-qPCR. Indeed, dUtx depletion leads to retention of H3K27me3 at DSBs in facultative heterochromatin, whereas the H3K27me3 levels at euchromatic DSBs remain unaffected upon DSB induction in the presence or absence of dUtx (**Fig.3D**). Loss of dUtx did not alter the levels of the DSB marker γH2Av, indicating that the retention of H3K27me3 at DR-*white* sites upon dUtx depletion is not due to inefficient cutting by I-SceI (**Fig.S3B**). These data suggest that dUtx mediates the removal of H3K27me3 at DSB sites specifically in facultative heterochromatin.

dUtx has been described to demethylate H3K27me3 to H3K27me1[43], which is a histone modification enriched at highly transcribed gene bodies[44]. However, we find no change in H3K27me1 levels at eu- or facultative heterochromatic DSBs when compared to undamaged loci (**Fig.S3C**). Yet, internal controls[45] revealed that our H3K27me1 antibodies, in contrast to the H3K27me3 antibody (**Fig.S3D**), displayed (10-60%) binding to other modifications (**Fig.S3E, F**). Although selectivity of the used antibodies was highest for H3K27me1 (**Fig.S3E, F)**, it is difficult to strongly conclude that H3K27me1 levels remain constant after DSB induction. Altogether, our chromatin analyses revealed that DSBs in facultative heterochromatin cause a local loss of H3K27me3, which is dependent on the histone demethylase dUtx.

DSB movement and HR repair in facultative heterochromatin depend on dUtx

We next hypothesized that the local loss of H3K27me3 at DSBs in facultative heterochromatin could be required to promote DSB movement outside polycomb bodies. To test this, we turned to *Drosophila* cells in culture which allow for in-depth visualization of repair processes in combination with RNAi-mediated depletions. We visualized the dynamics of the early HR protein ATR Interacting Protein (ATRIP) within polycomb bodies (ph-p domains) in the presence and absence of dUtx using live imaging of 5Gy gamma-irradiated *Drosophila* Kc cells (**Fig.4A**). ATRIP binds to RPA-coated ssDNA strands, which are produced early in HR during 5' to 3' end-resection of the DSB[46]. We find that upon irradiation of control cells, ATRIP foci appear inside polycomb bodies and rapidly move outside these domains within ten minutes (**Fig.4B, C, Fig.S4A**), which recapitulates our previous findings on Mu2 foci dynamics at facultative heterochromatic DSBs in larval tissues (**Fig.2C, D, F**). Interestingly, ATRIP gets recruited inside polycomb bodies upon irradiation in the absence of dUtx, suggesting that end-resection steps are independent of dUtx (**Fig.4D, E, Fig.S4B**). However, dUtx depletion did lead to a delay in the movement of ATRIP-coated DSBs outside polycomb bodies. In control cells, 75% of ATRIP foci move outside the polycomb bodies within 10 minutes, while in dUtx depleted cells only 58% move out within this timeframe (**Fig.4C**). In line with this, we find an increased accumulation of ATRIP foci within the ph-p domains (24% in control cells during the course of our imaging experiment, compared to 35% in dUtx-depleted cells) indicative of defects in DSB movement upon dUtx depletion (**Fig.4E**).

Together, these results suggest that the early steps of HR (end-resection, ATRIP recruitment) occur efficiently within polycomb bodies and are independent of H3K27me3 demethylation at the DSB site. However, dUtx-mediated demethylation of H3K27me3 is required for the subsequent DSB movement outside the polycomb body. To test whether this dUtx-dependent DSB movement is required for later repair steps in facultative heterochromatin, we employed our *in vivo* DR-*white* reporter system, which allows us to directly assess DSB repair pathway choice (HR/NHEJ) by sequencing repair products (**Fig.1A**). Strikingly, loss of dUtx in larvae revealed a 40-52% reduction in the proportion of HR repair products at two of the three facultative heterochromatic DSB sites, while euchromatic DSB repair products remained unchanged (**Fig.4F**). This reduction in HR repair was accompanied by an increase in NHEJ repair at facultative heterochromatic DSB sites. These changes in DSB repair pathway choice are not due to indirect cell-cycle effects, since dUtx depletion did not significantly affect cell cycle progression in the Fly-FUCCI system[47] (**Fig.S4C**). Interestingly, dUtx loss only resulted in decreased HR at two of the three insertion sites (fHet1, 2, not fHet3). The fHet3 integration shows loss of H3K27me3 at the DSB site (**Fig.3A**), but dUtx depletion does not affect HR at this site (**Fig.4F**), indicating that redundant mechanisms, independent of dUtx, are active at this fHet3 site and can promote HR. In contrast, DSBs at the fHet2 locus did not induce loss of H3K27me3 (**Fig.3A**) but did show a clear defect in HR in the absence of dUtx (**Fig.4F**). This suggests that dUtx does play a role in DSB repair at this integration site, but that we may not have been able to identify dUtx-mediated loss of H3K27me3 levels at these DSB sites due to relatively low sensitivity of our ChIP assay. Altogether, our results reveal that early HR steps in facultative heterochromatin can be performed in the absence of dUtx, and that dUtx is specifically required for DSB movement and completion of HR repair.

Finally, to assess the physiological role of dUtx in DSB repair in facultative heterochromatin, we determined whether survival of flies mutant for dUtx depends on the presence of intact DNA damage signaling. To do so, we crossed heterozygous dUtx mutant flies with flies that contain a truncation mutation in Ataxia telangiectasia and Rad3 related (ATR, mei41). ATR is one of the earliest kinases that rapidly respond to DSB events[48]. Combining dUtx heterozygous mutant flies with an ATR mutation reduces relative viability by 16% (from 62% absolute viability in dUtx mutant alone to 52% viability in dUtx mutant combined with ATR mutant), indeed suggesting that dUtx mutant flies depend on correct DNA damage repair signaling for their survival (**Fig.S4E**). Moreover, this result reveals that in the absence of proper facultative heterochromatic DSB repair, flies depend on ATR to maintain viability.

DISCUSSION

Chromatin forms dynamic domains in the nucleus, each characterized by specific molecular properties, which can directly influence the process of DSB repair. However, how transcriptionally inactive facultative heterochromatin (i.e. polycomb chromatin) influences DSB repair remains poorly understood. To address this question and understand how eukaryotic cells maintain the integrity of silenced developmental genes, we here integrated inducible single DSB systems in eu- and facultative hetero-chromatin in *Drosophila* animals. This work, for the first time, allows us to study DSB repair in detail in facultative heterochromatin in animal tissue. Using these systems, we find that DSBs in facultative heterochromatin rapidly move outside polycomb bodies within minutes after their appearance. This movement depends on the H3K27me3 demethylase dUtx. In line with this, we find evidence for dUtx-mediated loss of the silencing mark H3K27me3 near DSBs in facultative heterochromatin. Our data further reveal that early steps of HR (i.e. end-resection) can occur efficiently within polycomb bodies and are independent of dUtx, whereas dUtx is required to promote subsequent DSB movement and the completion of HR within facultative heterochromatin. Together, we propose a model in which resected DSBs in polycomb bodies are subjected to dUtx-mediated loss of the silencing mark H3K27me3, which in turn promotes DSB movement and timely repair by homologous recombination (**Fig.5**).

Specific movements of DSBs have been identified to occur in a variety of chromatin compartments including centromeres[16], nucleoli[17–19] and pericentromeric constitutive heterochromatin[16,20–22]. Movement of these DSBs has been suggested to promote binding of HR proteins[17,18], as well as prevent aberrant recombination between repetitive sequences[20,21]. Interestingly, we here for the first time find that DSBs move outside polycomb bodies *in vivo* (**Fig.2**). In contrast to centromeres, nucleoli and constitutive heterochromatin, facultative heterochromatin mainly contains unique sequences and is deprived of repetitive sequences. We therefore propose that DSB movement in facultative heterochromatin did not evolve to prevent aberrant recombination, but rather reflects the necessity to create a repair-competent state, facilitating access to the DSB repair machinery (**Fig.5**).

Our data suggest that the movement of DSBs outside polycomb bodies is directly regulated by the dUtx-mediated removal of H3K27me3 at the break site (**Fig.3, 4**). H3K27me3 is usually required to recruit PcG proteins to enhance compaction and maintain the silenced state[9–13]. Therefore, active removal of H3K27me3 at the DSB site by dUtx could directly lead to a local loss of PcG proteins. This can subsequently lead to changes in the molecular- and biophysical- properties of the DSB locus, creating an environment distinct from the surrounding polycomb body and the active expulsion or passive separation of the DSB from the polycomb body. In line with this hypothesis, we find that in the absence of dUtx, and the subsequent retention of H3K27me3, DSBs remain longer within the polycomb body (**Fig.4**). These results reveal analogies with our previous findings at DSBs in constitutive heterochromatin, where we found that loss of the silencing mark H3K9me3 at DSBs by the histone demethylase dKDM4A ensures DSB movement outside the constitutive heterochromatin domain[31,32].

Since we did not observe a complete inhibition of facultative heterochromatic DSB movement upon dUtx depletion, additional processes are likely involved, such as for example DSB end-processing or the recruitment of specific chromatin- or repair- proteins. End-resection as well as chromatin proteins, including the cohesin- and SMC5/6- complexes, were previously found to drive DSB movement in other chromatin domains[16,18,20], suggesting that additional components could be driving movements in polycomb bodies as well.

In addition to a role in DSB movement, our findings indicate that dUtx-mediated demethylation of H3K27me3 at facultative heterochromatic DSBs is important for repair pathway choice, since dUtx loss shifts the repair pathway choice towards NHEJ, resulting in decreased HR (**Fig.4**). Considering that dUtx depletion only affects DSB repair pathway choice in facultative heterochromatin, not euchromatin, these repair pathway changes are unlikely to be driven by indirect general defects in cell cycle progression or transcriptional regulation of repair genes[49]. In line with this, we did not find any defects in cell cycle progression in larval tissue depleted of dUtx (**Fig.S4**). Therefore, we hypothesize that the HR/NHEJ repair pathway choice at facultative heterochromatin could be directly regulated by dUtx through two non-mutually exclusive mechanisms: (1) defects in DSB movement, and (2) direct impact on binding of HR- or NHEJ- proteins at DSB sites.

In the first model, dUtx is promoting HR by moving the DSB to a more HR-prone chromatin state depleted of silencing marks. The movement might therefore specifically facilitate the access to 'late' HR repair proteins (e.g. Rad51 loading or helicases to resolve D-loops), usually excluded from the compact polycomb state. Moreover, moving an HR-proficient DSB away from the compact facultative heterochromatin might provide the required chromatin mobility necessary to perform homology search[50]. Indeed, we observe that loss of dUtx has no impact on the initial stages of homologous recombination (HR), such as end-resection and ATRIP loading, in polycomb bodies. However, dUtx loss does impede DSB movement, and likely subsequent later HR steps as evidenced by the decreased number of HR repair products identified at facultative heterochromatic DSB sites.

In our second model, we propose that the decreased frequency of HR repair in the absence of dUtx is a direct consequence of changes in binding of repair proteins to histone modifications at the break site. It is possible that the silencing mark H3K27me3 is able to directly bind specific NHEJ proteins within

the polycomb body. Alternatively, the H3K7me1 or unmethylated H3K27 residues, as a result of dUtx-mediated H3K27me3 demethylation, could directly recruit proteins important for HR. In line with this hypothesis, previous work identified that the HR-promoting TONSL-MMS2L complex has a higher affinity for unmodified histones, generated following DNA replication[51]. Moreover, previous research has identified that specific histone modifications can directly control binding of repair proteins. For example, H3K36me3, a histone modification associated with active transcription, has been found to recruit specific HR repair proteins through its reader protein LEDGF[14,52], while H4K16ac prevents binding of the NHEJ-promoting protein 53BP1[53]. Together, this suggests a strong impact of the loss and gain of specific histone modifications in direct repair protein recruitment.

Interestingly, here we find that loss of the silencing mark H3K27me3 promotes repair by HR. However, in constitutive heterochromatin we previously observed the opposite effect, where loss of the silencing mark H3K9me3 promotes repair by NHEJ[32]. This suggests that H3K27me3 and H3K9me3 have opposite impacts on DSB repair protein recruitment. Although both facultative- and constitutive-heterochromatin domains require DSB movement to ensure efficient HR, it now becomes clear that each of these domains require distinct histone modifying activities to ensure movement of these DSB as well as repair pathway choice.

Moreover, accumulation of H3K27me3 and polycomb group proteins at break sites in euchromatin have been suggested to be important for the local inhibition of transcription[54–56]. Since we find a specific loss of H3K27me3 only at facultative heterochromatin DSB sites, these findings together support our general model in which each chromatin domain requires differential chromatin changes to coordinate repair. While actively transcribed regions may require accumulation of H3K27me3, silenced regions may require its loss to overcome the barrier for repair.

Despite differences in dUtx-dependency for repair pathway choice, we do find that the frequencies of either HR- or NHEJ- repair pathway usage in facultative heterochromatin in wildtype tissue are similar to DSBs in euchromatin (**Fig.1, Fig.S1**). These results are consistent with previous findings in which DSBs in H3K27me3-enriched imprinted loci in mice did not differ in repair pathway usage when compared to the corresponding active allele[57]. In contrast, a recent study that used a sequencing-based reporter system in cancer cells to investigate the impact of chromatin on CRISPR-Cas9 induced DSBs did reveal differences in repair pathway usage in H3K27me3-enriched regions[58]. Using this system, the authors found a relative decrease in NHEJ and concurrently an increase in usage of Microhomology-Mediated End-Joining (MMEJ) within H3K27me3-enriched regions. MMEJ is an error-prone mechanism that relies on short-range end-resection and uses homologous sequences to align the broken ends. In contrast to our results, these findings suggests that end-resection-based repair pathways are preferred at facultative heterochromatic DSBs. These different outcomes could indicate differences in repair pathway usage between species or could be due to the approach used to induce DSB induction (CRISPR-Cas9 versus I-SceI). Moreover, in our *in vivo* DR-*white* system, facultative heterochromatin likely more closely resembles the physiological formation of polycomb bodies compared to tumor cells in culture.

Finally, the fact that we identify similar DSB repair pathway usage in eu- and facultative-heterochromatin implies that removal of H3K27me3 at facultative heterochromatic DSB sites could create a more euchromatin-like state. The appearance of an actively transcribed compartment to enhance DSB repair has recently been described to occur upon clustering of AsiSI-induced DSBs in euchromatin of human osteosarcoma cells (U2OS)[59]. It will be interesting to determine whether DSBs in polycomb bodies depend on a similar formation of an actively transcribed euchromatin- state, and whether additional chromatin changes are necessary to promote this state within this compact domain.

In conclusion, our work demonstrates that DSBs in facultative heterochromatin require specific local chromatin changes and DSB movement for their faithful repair in animal tissue. Our results emphasize the importance of understanding how different chromatin components influence DSB repair pathway choice and maintain genome stability across diverse chromatin domains. Facultative heterochromatin

regions are often associated with high mutational loads in cancer[60], indicating that these domains are particularly vulnerable to aberrant DNA damage repair. Moreover, the human homolog of dUtx, UTX, is often mutated in cancer[61]. In the long-term, research into DNA damage repair in heterochromatin will give insights into how misregulation of chromatin proteins, such as UTX, could result in increased genome instability and specific mutational signatures in cancer, ultimately contributing to disease development.

METHODS

Constructs

DR-*white* construct was created previously[22]. ph-p was N-terminally tagged with mCherry and cloned in a pCasper5 vector for random p-element transformation in flies. For cell culture experiments, ph-p and ATRIP were cloned into pCopia vectors containing N-terminal mCherry or C-terminal eYFP epitope tags, respectively. ph-p was cloned from the pFastBac plasmid (Addgene #1925), whereas ATRIP was cloned from cDNA generated from RNA extracted from wild type flies.

Fly lines

Flies were grown at room temperature on standard medium, except otherwise specified. Embryo injection and generation of new DR-*white* and ph-p-mCherry fly lines were performed by BestGene, Inc. DR-*white* attB containing plasmids were integrated in Minos-mediated integration cassette (MiMIC) integration sites as described previously[22]. Facultative heterochromatin integration sites were selected based on high H3K27me3 levels in OregonR flies (modEncode) and integration near a gene known to be regulated by H3K27me3 and/or polycomb group proteins[62]. Euchromatin integration sites were selected based on low H3K27me3 and H3K9me2/3 levels. To create ph-p-mCherry fly lines, pCasper5-ph-p-mCherry plasmid containing the copia promoter and P-element transposons was injected in embryos of w1118 flies. To induce knockdown of either CtIP or dUtx, flies containing Gal4 driven by an Actin5C promoter were crossed with UAS-CtIP RNAi or UAS-dUtx RNAi. A list of MiMIC integration sites to generate the DR-*white* fly lines as well as all fly lines used can be found in Supplemental Table S1.

ChIP-qPCR

Third instar DR-*white* larvae (+ and - hsp.I-SceI) were heat-shocked for 1 hour to express hsp.I-SceI. Six hours after I-SceI activation, 40 larvae per condition were pooled together to extract chromatin following the ChIP protocol of the Kevin White lab (https://www.encodeproject.org/documents/f890fde6-924c-4265-a60f-c5810401066d/), with slight adjustments. In short, larvae were homogenized in buffer A1 (60mM KCl, 15mM NaCl, 15mM HEPES pH7.6, 4mM MgCl2, 0.5% TritonX-100, 0.5mM DTT, protease inhibitor (Roche #1873580)) and fixed using 1.8% paraformaldehyde (EMS). Fixation was stopped by addition of glycine. After several washing steps with buffer A1, nuclei were isolated (140mM NaCl, 15mM HEPES pH7.6, 1mM EDTA, 0.5mM EGTA, 0.1% sodium deoxycholate, 1% TritonX-100, 500µM DTT, protease inhibitors, 0.1% SDS and 0.5% N-lauroylsarcosine) and the chromatin was fragmented by sonication for 10 cycles on Bioruptor (Diagenode high settings, 30'' on/off). Chromatin was separated from cell debris using centrifugation and stored at -80C for maximum of 2 months.

ChIP was performed as described earlier[63] using 1-2 ug chromatin and 1-5 ug antibody. ChIP antibodies used were mouse anti-γH2Av (Developmental Studies Hybridoma Bank, UNC93-5.2.1), rabbit anti-H3K27me3 (Invitrogen, MA5-11198), rabbit anti-H3 (Abcam, ab1791), and rabbit anti-H3K27me1 (Diagenode, C15410045-50 or Abcam, ab195492). Enrichment of the specific histone mark was quantified by qPCR using SYBR Green Master Mix (Roche) and qPCR primers 1.4 kb away from the DSB (3xp3) as well as primers for an internal control. All qPCR primer sequences are listed in Supplemental Table S1.

We used Epicypher SNAP-ChIP K-MetStat panel (19-1001) to validate the specificity of the antibodies used for ChIP (**Fig.S3D-F**). The SNAP-ChIP panel contains barcoded nucleosomes with a specific methylation mark (unmethylated H3, H3K4me1/me2/me3, H3K9me1/me2/me3, H3K27me1/me2/me3,

and H3K36me1/me2/me3). 0.4µL of 0.6 nM K-MetStat stock per 2 ug of chromatin was added at the start of the ChIP procedure. Subsequent qPCR analysis using primers specific to unique barcodes corresponding to each modified nucleosome in the panel allows the quantification of antibody specificity and efficiency.

DR white repair product analysis

Quantification of somatic repair products in DR-*white*, I-SceI larvae was performed as described previously [22]. In short, either hsp.I-SceI was expressed using heat-shock in third instar DR-*white*/hsp.I-SceI larvae, 24 hours before single larvae were collected. Alternatively, ecDHFR-I-SceI was expressed by feeding DR-*white*/ecDHFR-I-SceI 80µM trimethoprim (Sigma) throughout development (3-4 days), thereby stabilizing the ecDHFR-I-SceI protein. To prepare trimethoprim containing food, 1.67g instant *Drosophila* medium (Formula 4-24, Carolina Biological Supply) was mixed with 5 mL non distilled water containing 5.3 uL of 100 mM trimethoprim while vortexing.

To analyze repair products, the upstream *white* gene was PCR amplified and the PCR product was treated with ExoSAP-IT to enzymatically remove excess primers and unincorporated nucleotides, followed by Sanger sequencing. Analysis of Sanger sequences was performed using the TIDE (tracking of indels by decomposition) algorithm[35]. HR repair products were identified by loss of the I-SceI cleavage site and appearance of the wildtype *white* gene, which is essentially a 23-nucleotide deletion at the I-SceI cut site. NHEJ products were identified as insertions and deletions up to 25bp, except for the 23-nucleotide deletion. For PCR and sequencing primers, see Supplemental Table S1.

IF staining

Wing discs were dissected from third instar DR-*white* larvae and fixed on slides as described earlier[64] . Slides were stored in 96% ethanol at -20°C until staining procedure. Slides were thawed at room temperature and washed in PBS for 20 minutes. Tissues were blocked using 0.4% Triton in PBS and 5% milk for 1 hour at room temperature. Primary antibody incubations were performed overnight at 4°C in block buffer. Primary antibody used for imaging was mouse anti-γH2Av (1:250, Developmental Studies Hybridoma Bank, UNC93-5.2.1). Slides were washed 3 times with block buffer. Secondary antibody incubation was performed at room temperature in PBS 0.4% Triton for 2 hours. Secondary antibody used was Alexa 568 goat anti-mouse (1:600; Invitrogen). Slides were subsequently washed 3 times with 0.4% Triton in PBS, incubated with 3µg/ml DAPI for 30 minutes, washed with PBS and mounted using Prolong Diamond Antifade Mountant and a 20x20 mm #1.5 coverslip.

RT-qPCR

RNA was isolated by homogenizing either single larvae or Kc cells in 200µL Trizol (Invitrogen) using an electrical douncer (VWR). After addition of 40µL of chloroform and centrifugation, RNA from the aqueous phase was precipitated using isopropanol and further purified using an ethanol wash step. cDNA was synthesized using iScript following standard cDNA synthesis protocol (Bio-Rad). qPCR was subsequently performed on cDNA with gene-specific primers and control primers (Supplemental Table S1).

Cell culture

Kc cells were cultured in CCM3 medium (Avantor) supplemented with Antibiotic Antimycotic Solution (Sigma) at 27°C. To induce expression of ph-p-mCherry and ATRIP-eYFP, Kc cells were transiently transfected with 400ng of each plasmid using the TransIT-2020 reagent (Mirus). Live imaging was performed 72 hours after transfection.

To induce RNAi-mediated depletion of dUtx, cells were transfected with 5ug dsRNA (TransIT-2020 reagent (Mirus)) and harvested or subjected to imaging 3 days later. dsRNA was generated using a MEGAScript T7 transcription kit (Life Technologies). PCR products containing T7 promoter sequences and the target regions were used as templates (Supplemental Table S1).

Imaging

Images of fixed tissue, Fly-FUCCI wing discs and transfected Kc cells were acquired using a 60x oil immersion objective (NA 1.42) on a DeltaVision microscope (DeltaVision Spectris; Applied Precision, LLC). For all DSB tracking experiments in wing discs, time-lapse images were acquired using a LD C-Apochromat 63x/1.15 W Korr M27 objective on a LSM880 microscope with Airyscan (Zeiss), and images were processed using the Zeiss ZEN software. Time-lapse images were acquired once every 5-10 minutes. Image analysis and focus tracking were performed manually using the Fiji image analysis software.

For live imaging of wing discs, third instar larvae were dissected, and wing discs were placed on a slide in 10 µL of Schneider S1 medium supplemented with 10% FBS and covered with a 20×20 mm #1.5 coverslip as described previously[65]. To induce ecDHFR-I-SceI in larval tissue, 400 µM trimethoprim (Sigma) was added 15 minutes prior to imaging.

ACKNOWLEDGEMENT

We thank the Lens and Janssen lab members for their valuable input during laboratory meetings. Special thanks to Johannes Lehman for his input on visualizing live imaging data. This work was funded by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program, grant agreement No. 850405, and VIDI VI.Vidi.203.001 financed by the Dutch Research Council (NWO).

FIGURE 1

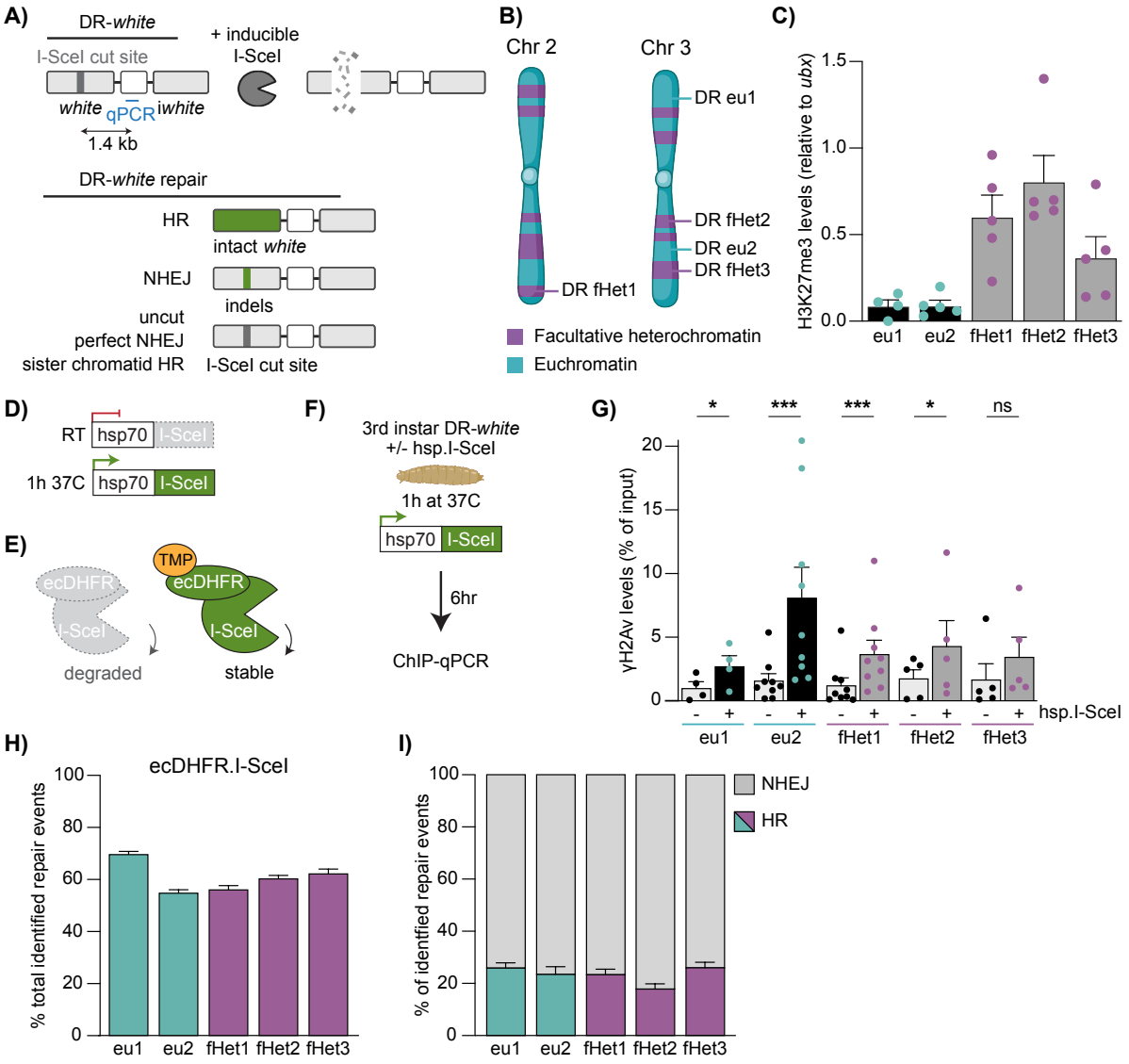

Figure 1. DR-*white* system to induce single DSBs in euchromatin and facultative heterochromatin.
A) Schematic of the DR-*white* system. Inducible expression of I-SceI creates a single DSB in the upstream *white* gene. HR with the downstream i*white* sequence results in loss of the I-SceI cut site (-23bp), and thereby an intact upstream *white* gene. NHEJ can result in indels at the DSB site. Perfect NHEJ or HR using the sister chromatid can lead to recreation of the I-SceI cut site. **B**) Schematic of DR-*white* integration sites in the fly genome (2x in euchromatin [DR *eu1*, DR *eu2*] and 3x in facultative heterochromatin [DR *fHet1*, DR *fHet2*, DR *fHet3*]). **C**) ChIP-qPCR analysis for H3K27me3 at the DR-*white* locus using qPCR primers that bind 1.4kb downstream of the I-SceI cut site (indicated in Fig 1A). H3K27me3 levels were normalized using *ubx* gene qPCR primers as a positive internal control (*ubx* has consistently high H3K27me3 levels). Averages are shown for ≥4 independent experiments +SEM. **D**) Schematic of the hsp.I-SceI construct. The hsp70 promoter upstream of I-SceI can be activated by shifting larvae for one hour to 37°C. RT = room temperature. **E**) Schematic of the ecDHFR-I-SceI system. Proteasomal degradation of ecDHFR-I-SceI can be blocked by adding the stabilizing compound trimethoprim (TMP). **F**) Experimental set-up for ChIP-qPCR experiments upon DSB induction using hsp.I-SceI. 3rd instar DR-*white* larvae with and without hsp.I-SceI

(control) were heat-shocked for one hour at 37°C to activate the *hsp70* promoter. Six hours later chromatin was extracted from 40 larvae per condition and subjected to ChIP-qPCR using the qPCR primers indicated in Fig 1A. **G**) ChIP-qPCR analysis for γH2Av in the absence (-) and presence (+) of hsp.I-SceI (as in Fig 1F). **H, I**) DR-*white*/ecDHFR-I-SceI larvae were fed trimethoprim (TMP) for 3-4 days to induce I-SceI. Repair products at the upstream *white* gene were PCR amplified, Sanger sequenced and analyzed using the TIDE algorithm[35]. Graphs show quantification of total identified repair events in all PCR products (**H**) and the percentage of repair products with either HR (green/purple) or NHEJ (grey) signatures (**I**). Bars indicate averages +SEM of ≥9 independent experiments (larvae) per condition. (ns) not significant, (*) p-value≤0.05, (***) p-value≤0.001, ratio paired t-test (**G**).

FIGURE 2

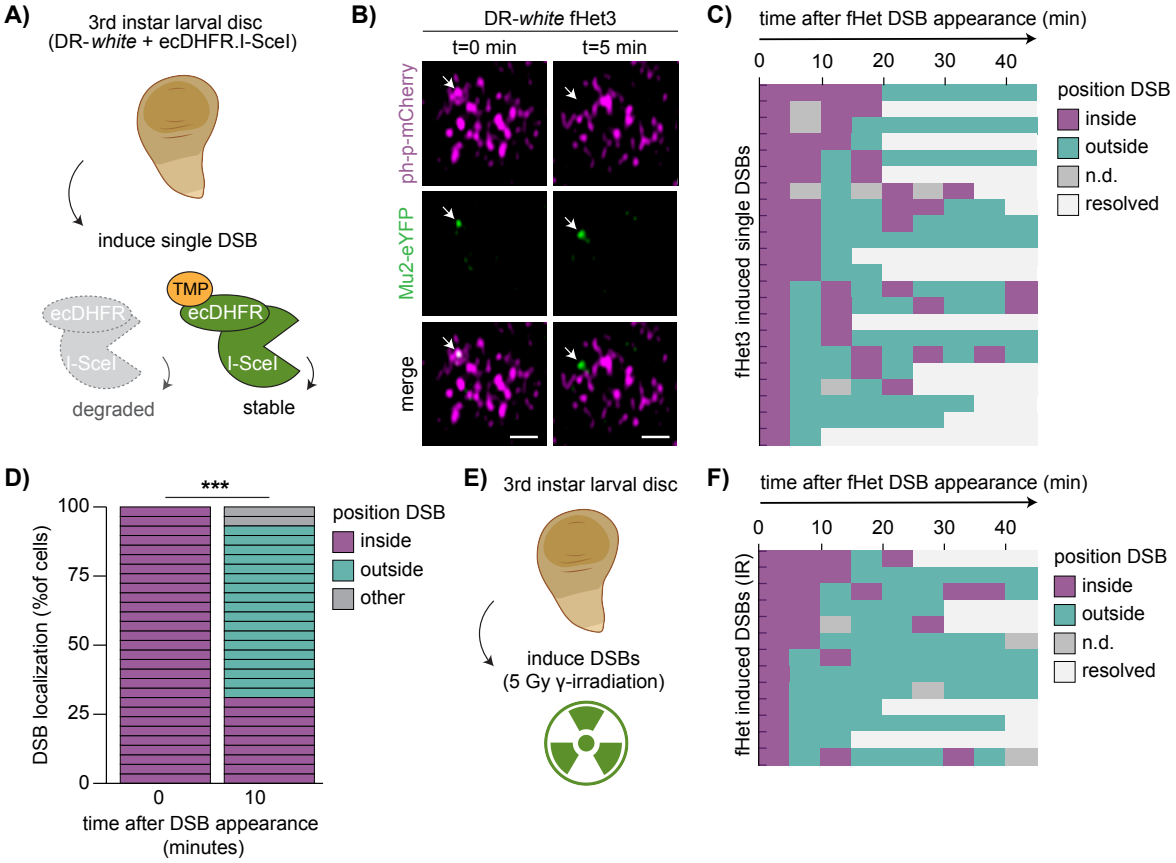

Figure 2. DSBs in facultative heterochromatin move outside polycomb bodies.
A) Set-up to analyze the dynamics of a single DSB in larval tissue. Wing discs of DR-*white*/ecDHFR-I-SceI 3rd instar larvae were dissected and placed in medium with trimethoprim (TMP) to stabilize ecDHFR-I-SceI. **B**) Representative images of Mu2-eYFP focus dynamics (DSB marker, green) within the ph-p-mCherry domain (polycomb marker, magenta) in 3rd instar larval wing disc. Arrow indicates Mu2-eYFP focus arising within ph-p-mCherry domain (0 min) and moving outside this domain (5 min). Scale bar = 1µm. **C**) Quantification of live imaging of Mu2-eYFP dynamics and kinetics over time relative to the ph-p-mCherry domain using single DSB induction (ecDHFR-I-SceI, *fHet*3). Each row indicates one single Mu2-eYFP focus that appeared within a polycomb body. Mu2-eYFP foci were followed up to 45 minutes after appearance. Colors indicate localization of the Mu2-eYFP focus (inside polycomb body [purple], outside [green], not determined [grey], or resolved [white]). **D**) Quantification of Mu2-eYFP localization 10 minutes after appearance in ph-p domains. **E**) Set-up to analyze the dynamics of DSBs using irradiation. Wing discs of 3rd instar larvae were dissected and placed in medium, followed by 5Gy gamma-irradiation. **F**) Quantification of live imaging of Mu2-eYFP dynamics and kinetics over time using 5Gy irradiation (quantification as in **C**). (***) p-value≤0.001, Chi-square test (**D**).

FIGURE 3

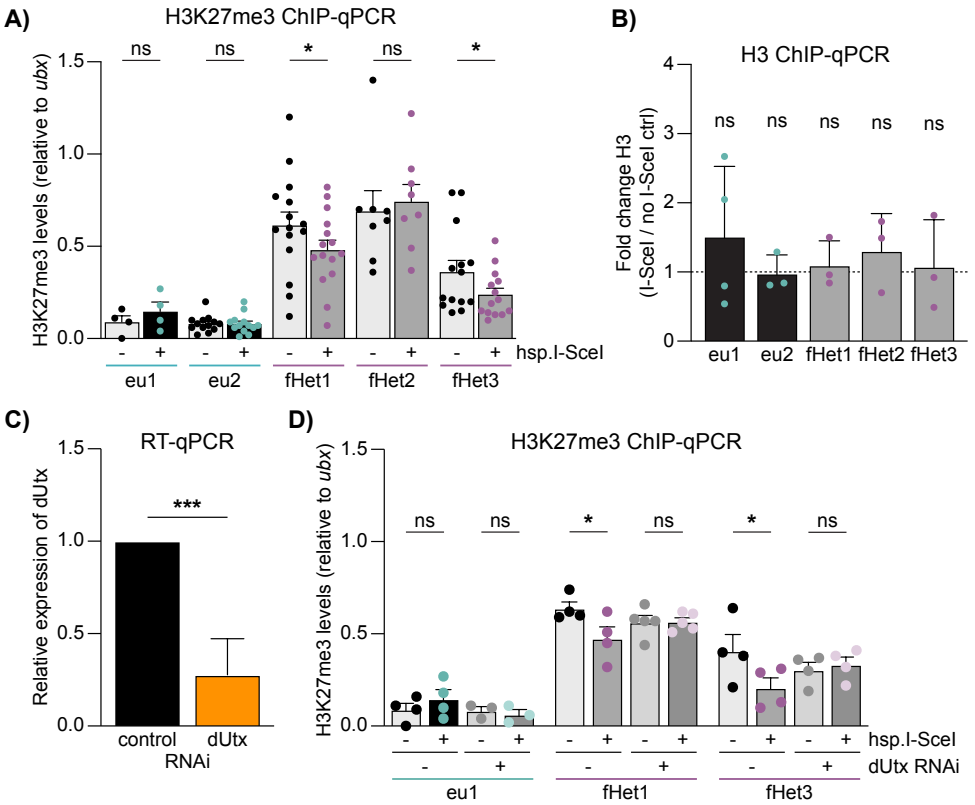

Figure 3. Loss of H3K27me3 at facultative heterochromatic DSBs is mediated by dUtx.

A) ChIP-qPCR analysis for H3K27me3 in the absence (-) and presence (+) of hsp.I-SceI (as in Fig 1F) at indicated DR-*white* integration sites. H3K27me3 levels were normalized using a *ubx* qPCR primer set as an internal positive control (*ubx* has consistently high H3K27me3 levels). Averages are shown for ≥4 independent experiments +SEM. **B**) ChIP-qPCR analysis for histone H3. Fold change indicates H3 levels at DR-*white* locus in hsp.I-SceI-expressing larvae (+DSB) divided by larvae not expressing hsp.I-SceI (-DSB). Dotted line indicates no change (fold change = 1). H3 levels were normalized using a *yellow* qPCR primer set as an internal positive control. Bars indicate fold change +SD of ≥3 independent experiments. **C**) Relative dUtx expression level in 3rd instar larvae normalized to an internal control gene (tubulin), determined using RT-qPCR. Bars indicate averages +SD of 8 single larvae per condition (luciferase control RNAi or dUtx RNAi). **D**) ChIP-qPCR analysis for H3K27me3 in the absence or presence of a DSB (-/+ hsp.I-SceI), with or without dUtx RNAi depletion (as in Fig 1F). H3K27me3 levels were normalized using a *ubx* qPCR primer set as internal positive control. Averages are shown for ≥4 independent experiments +SEM. (ns) not significant, (*) p-value≤0.05, (***) p-value ≤0.001, paired t-test (**A, B, D**) and unpaired t-test (**C**).

FIGURE 4

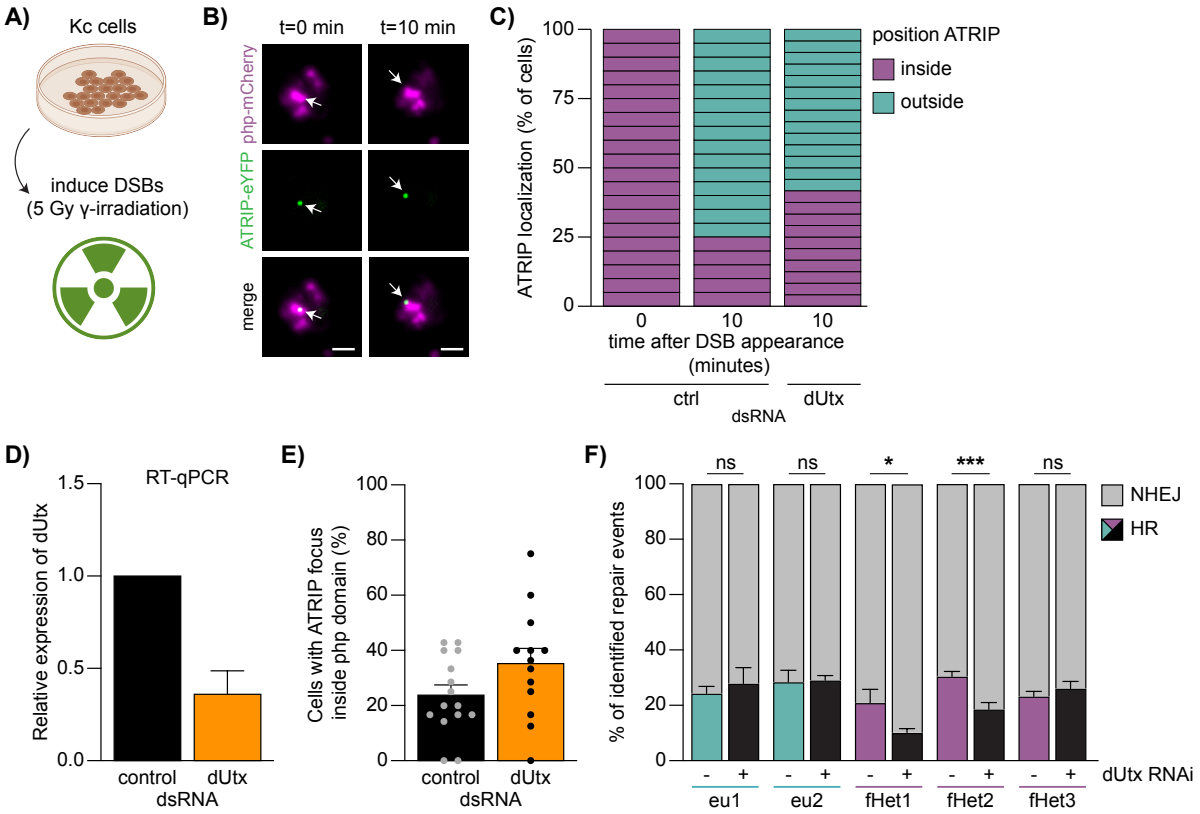

Figure 4. DSB movement and HR repair in facultative heterochromatin depend on dUtx.
A) Cell culture set-up to analyze the dynamics of ATRIP localization upon DSB induction using 5Gy gamma-irradiation. **B**) Representative images of ATRIP-eYFP focus (green) dynamics within the ph-p-mCherry domain (magenta). Arrow indicates ATRIP-eYFP focus arising within ph-p-mCherry domain (0 min) and moving outside this domain (10 min). Scale bar = 2µm. **C**) Quantification of ATRIP-eYFP localization 10 minutes after ATRIP-eYFP appearance. **D**) Relative dUtx expression level in *Drosophila* Kc cells normalized to an internal control gene (tubulin), determined using RT-qPCR. Bars indicate averages +SD of 3 independent experiments per condition (yellow control dsRNA or dUtx dsRNA). **E**) Quantification of the number of cells with an ATRIP-eYFP focus inside ph-p-mCherry domain divided by the total number of cells expressing both ATRIP-eYFP and ph-p-mCherry. Each dot indicates the percentage of cells with ATRIP foci in ph-p per acquired image. Bars indicate averages +SEM of 3 independent experiments condition (≥6 acquired images). **F**) DR-*white*/hsp.I-SceI larvae with (+) or without (-) dUtx RNAi were heat-shocked for one hour to induce I-SceI. Repair PCR products were Sanger sequenced 24 hours after I-SceI induction and analyzed using the TIDE algorithm. Graph shows percentage of identified HR (color, black) and NHEJ products (grey). Bars indicate averages +SEM of ≥5 independent experiments (larvae) per condition. (ns) not significant, (*) p-value≤0.05, (***) p-value≤0.001, unpaired t-test (**D, E, F**).

FIGURE 5

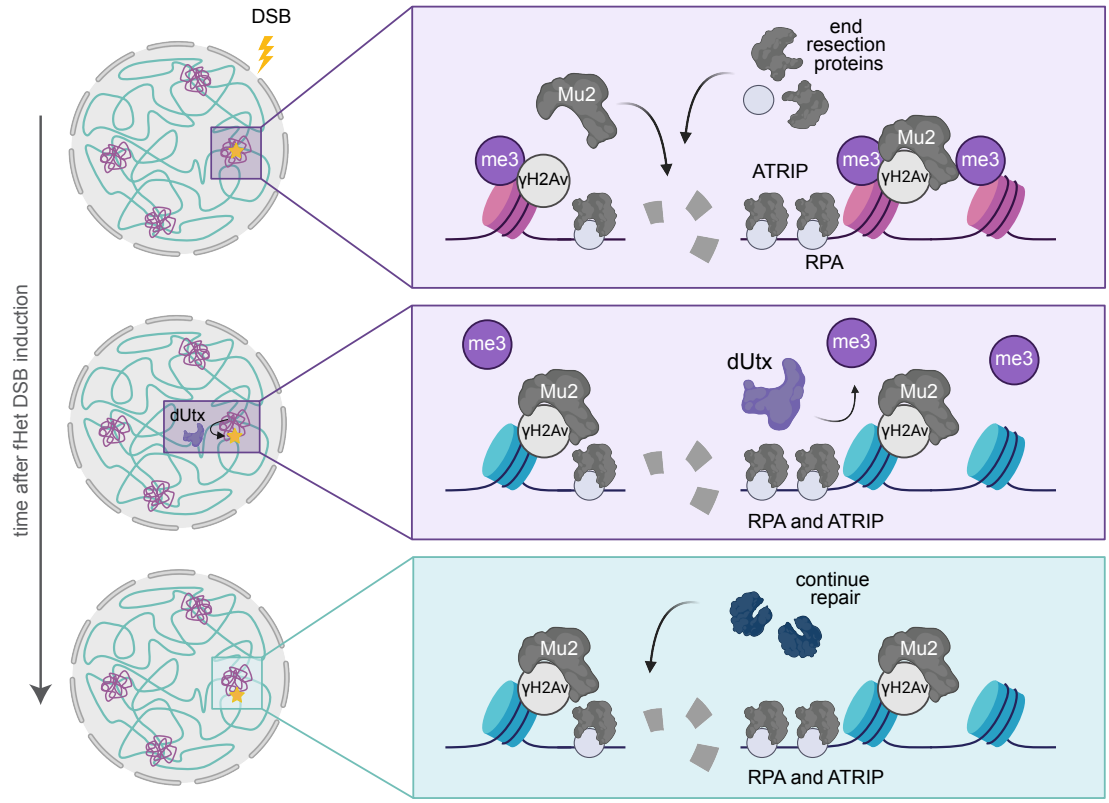

Figure 5. Model

Model for facultative heterochromatin DSB repair. Facultative heterochromatin (purple) and euchromatin (blue) form distinct domains within the nucleus. Facultative heterochromatin is characterized by H3K27me3 and accumulates in polycomb bodies. DSBs in facultative heterochromatin undergo early steps of HR (i.e. Mu2-recruitment, end-resection) within polycomb bodies. dUtx is required to demethylate H3K27me3 the DSB site and promote the subsequent DSB movement. These processes are necessary to efficiently repair DSBs with HR within facultative heterochromatin.

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