The Mre11-Rad50-Nbs1 complex mediates the robust recruitment of Polo to DNA lesions during mitosis

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Keywords: Mitosis, Drosophila, DNA damage, Polo, BubR1, Bub3, Mre11, Rad50, Checkpoint, Chromosomes, anaphase, ACP/C, Cdc20, DNA double strand breaks
Abbreviations

APC/C: anaphase promoting complex/cyclosome
ATM: ataxia-telangiectasia mutated
BRCT: BRCA1 C-terminal
BRCA1: Breast cancer type 1 susceptibility protein
CDK1: cyclin-dependent kinase 1
CtIP: C-terminal Binding Protein Interacting Protein
DDR: DNA damage response
DSB: double strand break
HR: homologous recombination
IR: irradiation
MDC1: Mediator of DNA Damage Checkpoint Protein 1
MRE11: Meiotic recombination 11 homolog 1
NBS1: Nijmegen breakage syndrome protein 1
NHEJ: non-homologous end joining
PBD: Polo box domain
PIKK: phosphoinositide-3-kinase-related kinases
PLK1: polo-like kinase 1
SAC: Spindle Assembly Checkpoint
Summary statement

Landmann et al. measure the kinetics of the DNA damage sensor Mre11 and the mitotic proteins Polo, BubR1, Bub3 at DNA lesions during mitosis and reveal their order of recruitment.
Abstract

The DNA damage sensor, Mre11-Rad50-Nbs1 complex, and Polo kinase are recruited to DNA lesions during mitosis. However, their mechanism of recruitment is elusive. Here, using live-cell imaging combined with the micro-irradiation of single chromosomes, we analyze the dynamics of Polo and Mre11 at DNA lesions during mitosis. The two proteins display distinct kinetics. While Polo kinetics at DSBs are Cdk1-driven, Mre11 promptly but briefly associates with DSBs regardless of the phase of mitosis and re-associates with DSBs in the proceeding interphase. Mechanistically, Polo kinase activity is required for its own recruitment and that of the mitotic proteins BubR1 and Bub3 to DSBs. Moreover, depletion of Rad50 severely impaired Polo kinetics at mitotic DSBs. Conversely, ectopic tethering of Mre11 to chromatin is sufficient to recruit Polo. Our study highlights a novel pathway that links the DSB sensor MRN complex and Polo kinase to initiate a prompt, decisive response to the presence of DNA damage during mitosis.
Introduction

Cells encounter genotoxic stress originating from extrinsic and intrinsic sources (Cannan and Pederson, 2016). DNA double strand breaks are potentially the most harmful damage as they can cause genomic instability, a hallmark of cancer cells (Jackson and Bartek, 2009). The presence of DSBs in interphase triggers a conserved signaling pathway, also known as the DNA Damage Response (DDR), that promotes DNA repair concomitantly with cell cycle delay through checkpoint activation (Hartlerode and Scully, 2009; Melo and Toczyski, 2002; Sekelsky, 2017). In brief, DSBs are promptly recognized by DNA damage sensors including the conserved proteins Mre11 and Rad50, which are part of a complex with the less conserved Nbs1 protein (also called Nbx) (Lisby et al., 2004; Lukas et al., 2003; Lukas et al., 2004; Syed and Tainer, 2018; Williams et al., 2010). Subsequently, the Mre11/Rad50/Nbs1 (MRN) complex recruits the phosphoinositide-3-kinase-related kinases (PIKK) Ataxia Telangetasia Mutated (ATM) to the site of DNA damage via direct interactions (Falck et al., 2005). ATM promotes the rapid modification of chromatin flanking the breaks, including extensive phosphorylation of the histone 2A variant H2AX (Rogakou et al., 1998), which serve as docking sites for BRCT motifs present in Mediator of DNA damage Checkpoint protein 1 (MDC1) (Dronamraju and Mason, 2009; Stewart et al., 2003; Stucki et al., 2005). MDC1 directly interacts with Nbs1 and recruits additional MRN complexes to DSBs via an amplification loop (Lukas et al., 2004; Wu et al., 2008). This primary signal is transduced to effectors that direct DNA repair through two main pathways: the error prone non-homologous end-joining pathway (NHEJ) or the error-free homologous recombination pathway (HR) (Ceccaldi et al., 2016; Syed and Tainer, 2018).
Studies have reported a variety of responses to the presence of DSBs during mitosis depending on the stage at which the damage occurred (Blackford and Stucki, 2020; Thompson et al., 2019). A single broken chromosome end created by the breakage of a dicentric chromosome during anaphase can produce a chromosomal breakage-fusion-bridge cycle (McClintock, 1938). It can also be repaired by either de novo telomere formation or homolog-dependent restoration of the chromosome terminus (Ahmad and Golic, 1998; Bhandari et al., 2019; Haber and Thorburn, 1984; McClintock, 1939; McClintock, 1941). In budding yeast, a DSB created during telophase triggers reversion of chromosome segregation and sister chromatids coalescence that facilitates repair by HR (Ayra-Plasencia and Machin, 2019). In vertebrates, the presence of DSBs in prometaphase activates primary DNA damage signaling, comprising of DNA lesion recognition by the MRN complex, followed by the partial activation of ATM and subsequent H2AX phosphorylation on chromatin proximal to the breaks (Benada et al., 2015; Giunta et al., 2010; Gomez-Godinez et al., 2010; Orthwein et al., 2014; Peterson et al., 2011; Silva et al., 2014; Terasawa et al., 2014). Subsequent downstream signaling pathways that promote DNA repair by NHEJ and HR are inhibited until the next G1 phase (Benada et al., 2015; Giunta et al., 2010; Orthwein et al., 2014; Peterson et al., 2011; Terasawa et al., 2014; van Vugt et al., 2010; Yu et al., 2012). In Drosophila neuroblasts, clustered DSBs on one chromosome are recognized by the mitotic proteins Polo, BubR1 and Bub3. These proteins mediate the proper segregation of the damaged chromatid by tethering the two broken ends (Derive et al., 2015; Royou et al., 2010).

BubR1 and Bub3 act together to stabilize kinetochore-microtubule attachments. They also participate in the spindle assembly checkpoint (SAC), which inhibits the anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that triggers anaphase by
targeting key substrates for proteolysis, until all chromosomes are properly attached to the spindle (Basu et al., 1999; Basu et al., 1998; Karess et al., 2013; Lopes et al., 2005). In a recent study, we provided evidence that BubR1 and Bub3 depend on each other to localize on DNA lesions during mitosis where they promote the local inhibition of the APC/C via the sequestration of the APC/C cofactor Cdc20 (Fzy in Drosophila) (Derive et al., 2015).

Polo is a serine/threonine kinase that plays multiple conserved functions during mitosis including centrosome maturation, bipolar spindle formation, kinetochore function and cytokinesis (Archambault et al., 2015; Llamazares et al., 1991; Sunkel and Glover, 1988). We found that it participates in a signaling pathway that tethers chromosome fragments during mitosis, thus preventing genome instability (Royou et al., 2010). However, the molecular pathway governing Polo, BubR1 and Bub3 recruitment to broken chromosomes and their dependency relationship with the DNA damage signaling pathway identified in vertebrate mitotic cells has not been characterized.

In this study, we examined the kinetics of Polo, BubR1 and Bub3 at DNA lesions created with a pulsed-UV laser in a precise spatiotemporal manner during the different phases of mitosis to assess the coordination of their recruitment to DSBs and investigated their interplay with the DSB sensor, the MRN complex.
Results

Polo is promptly recruited to DNA lesions during mitosis and its kinetics are coupled to Cdk1 activity

The kinetics of Polo recruitment to DSBs in mitosis were first examined by inducing surgical damage to one chromosome in live Drosophila neuroblasts at precise times during mitosis with a pulsed-UV laser. The signal of GFP::Polo on the resulting DNA lesions was subsequently monitored by time-lapse imaging (within 1 to 5 seconds following irradiation). This method of micro-irradiation (IR) creates complex DNA lesions including DSBs (Alekseandrov et al., 2018). We first calibrated laser power to determine the optimal irradiation (IR) dose for our experiments. We identified 20% laser power as the lowest dose that produced a 100% response (i.e. the presence of a detectable Polo signal at the site of damage)(Fig. S1). In contrast to 20% laser power, lower IR doses resulted in a dramatic decrease in the frequency of cells exhibiting a detectable GFP::Polo signal at the lesions and a significant delay in the appearance of GFP::Polo, accompanied by reduced levels of GFP-Polo at the site of damage (Fig. S1). Since no noticeable changes in GFP::Polo kinetics were observed between 20 and 30% power, we chose to induce DSBs using 20% laser power in all subsequent experiments. This response was specific to damage on the chromosomes since no GFP::Polo foci were detected in the cytoplasm after IR (Fig. S1E).

Since Polo is targeted to different subcellular structures depending on the stage of mitosis, we reasoned that its recruitment to DSBs could be dependent upon the stage of mitosis during which DSBs were created. We therefore measured the kinetics of GFP::Polo on DSBs induced at different phases of mitosis. Cells were irradiated in prometaphase (after nuclear envelope breakdown and at least two minutes before anaphase onset), metaphase
(less than two minutes before anaphase onset), anaphase (within two minutes after anaphase onset) and telophase/G1 (>6 min after anaphase onset, during chromatin decondensation and nuclear expansion). Following IR, Polo signal was consistently detected at the site of DNA damage when the damage was induced during prometaphase, metaphase and anaphase (Fig. 1A). In contrast, either no GFP::Polo signal or traces were detected at the site of IR induced at telophase/G1 phase (T/G1)(Fig1A, C, F). In most cases, Polo exhibited biphasic kinetics at DSBs, with a period of accumulation followed by a period of dissociation. In some instances, a short plateau was observed between the phases of association and dissociation (Fig. 1B, PM curve). Polo signal was detected on DSBs 20 to 40 seconds following IR during all stages except T/G1 (Fig. 1C). Its half-time recruitment was variable (ranging from 1 to >6 min), particularly when IR was induced during prometaphase (Fig. 1D), and was correlated with the time elapsed between IR and anaphase onset (Fig. S2A, R²=0.5).

Importantly, Polo onset of dissociation from the damaged chromatin consistently occurred near or after anaphase onset, in concert with Cdk1 inhibition (Fig. 1B, E). The maximum levels of Polo at DNA lesions varied between cells, but did not correlate with the time elapsed between IR and anaphase onset (Fig. 1F and Fig. S2B). However, Polo never reached high levels on lesions induced after anaphase onset (Fig. 1F). We noted that a pool of Polo remained associated with DSBs more than 6 minutes following anaphase onset, which corresponds to the average time of completion of nuclear envelope reformation in Drosophila neuroblasts (Fig. 1A, last images of each panels)(Montembault et al., 2017). We conclude that Polo is rapidly recruited to DNA lesions encountered at any point during mitosis and that the efficiency of its recruitment to DSBs correlates with Cdk1 activity.
Since Polo dissociation from DNA lesions consistently occurred after sister chromatid separation, at a time of Cdk1 inhibition, we determined if Polo maintenance at DSBs correlated with the level of Cdk1 activity. To do so, we analyzed Polo signal in cells arrested in prometaphase with high Cdk1 activity as a result of colchicine treatment, which rapidly depolymerizes microtubules in mitotic neuroblasts (Fig. S3). We observed that the first phase of Polo association at lesions was similar between untreated and colchicine-treated cells (Fig. 1G, H). No significant differences in the half-time of Polo recruitment and its maximum levels at DNA lesions were observed between control and prometaphase-arrested cells (Fig. 1I, J). These results indicate that a functional spindle is not required for the efficient recruitment of Polo to DNA lesions. Interestingly, while Polo underwent a phase of dissociation from the lesions when untreated cells entered anaphase, Polo levels remained high and constant on the lesions in prometaphase-arrested cells (Fig. 1H). These results are consistent with a model in which Cdk1 activity is required for the maintenance of Polo at mitotic DSBs. Collectively, these results demonstrate the highly dynamic nature of Polo localization during mitosis, when it can be readily recruited to DNA lesions upon demand.

**Inhibition of Polo kinase activity affects its kinetics at DNA lesions**

The C-terminus of Polo contains two Polo Box Domains (PBDs) that target Polo to different sub-cellular structures, such as centrosomes and unattached kinetochores, via recognition of phosphoepitopes that serve as docking sites to the PBDs (Elia et al., 2003a; Elia et al., 2003b) (for review see (Schmucker and Sumara, 2014)). These phosphoepitopes on Polo targets are generated by priming kinases such as Cdk1, or by Polo itself (Kang et al., 2006; Lenart et al., 2007). This prompted us to determine if Polo kinase activity is required for its
own recruitment to DNA lesions. To do so, we treated cells with BI2536, an inhibitor of Polo kinase activity (Lenart et al., 2007; Riparbelli et al., 2014; Steegmaier et al., 2007). Polo inhibition is known to result in prolonged prometaphase (Conde et al., 2013; Lenart et al., 2007; Llamazares et al., 1991; Sumara et al., 2004; Sunkel and Glover, 1988). Therefore, we compared GFP::Polo kinetics at DNA lesions in cells treated with BI2536 with its kinetics in cells arrested in prometaphase after colchicine treatment, since colchicine did not affect the association phase of Polo at DSBs (as shown in Fig. 1H, I). We found that Polo inhibition using BI2536 severely altered GFP::Polo kinetics at DSBs (Fig. 2A, B). The initiation and the half-time of GFP::Polo recruitment to DNA lesions were delayed more than three-fold upon BI2536 treatment (Fig. 2C, D). Similarly, BI2536-treated cells did not display the high levels of GFP::Polo on lesions observed in untreated cells or after colchicine treatment (Fig. 1J, 2E). Cells treated simultaneously with colchicine and BI2536 exhibited similar defects in Polo kinetics at DSBs to cells treated with BI2536 alone (Fig. 2A-D). Our results indicate that the rapid and robust recruitment of Polo to DNA lesions depends on its kinase activity, as has been reported for its localization to centrosomes and kinetochores in human cells (Lenart et al., 2007).

BubR1, Bub3 and Fzy display biphasic kinetics at DNA lesions during mitosis.

Previously, we reported that BubR1 and Bub3 localized as a complex to damaged chromatids throughout mitosis, where they are required to sequester Fzy via its direct interaction with the KEN motif in BubR1 (Derive et al., 2015). We therefore analyzed the kinetics of GFP-tagged BubR1, Bub3 and Fzy after micro-irradiation (IR), as described for GFP::Polo. We observed that GFP::BubR1, GFP::Bub3 and GFP::Fzy were efficiently recruited to DNA breaks when the damage occurred before anaphase (Fig. 3A). Their kinetics on DNA lesions was
similar to GFP::Polo. All three proteins underwent a phase of association, culminating a few minutes after anaphase onset, followed by a phase of dissociation during anaphase/telophase (Fig. 3B, E). BubR1, Bub3 and Fzy shared similar timing of recruitment, which is consistent with their association to DSBs as a complex. However, their kinetics differed from Polo in three ways: first, their initiation and half-time of recruitment at the site of DNA damage were 4 and 3 times slower, respectively, compared with Polo (Fig. 3C and D). Second, unlike Polo, all three proteins dissociated completely from DBSs at telophase (Fig. 3B). Third, these proteins failed to localize to DNA lesions in more than half of the cells when the damage was induced after anaphase onset (Fig. 3A, % under right panels). This latter observation may be related to their slower rate of recruitment to DNA lesions, combined with their rapid and complete dissociation at telophase.

**Polo precedes BubR1 on DNA lesions and, while BubR1 completely dissociates from the lesions at telophase, a pool of Polo persists until interphase.**

The kinetics of Polo and the BubR1/Bub3/Fzy complex relative to each other at DSBs were monitored using simultaneous live cell imaging of GFP::BubR1 and mCherry::Polo after irradiation (Fig. 4). In all instances, we observed that GFP::BubR1 foci appeared after mCherry::Polo at the site of damage (Fig. 4A-D). Consistently, the half-time of BubR1 recruitment was delayed compared with Polo (Fig. 4E). Both proteins initiated their phase of dissociation after anaphase onset (except in one cell for mCherry::Polo)(Fig. 4F). However, while BubR1 completed its dissociation at telophase, a pool of Polo remained on the lesions and disassembled at a later stage during the next interphase (Fig. 4G). Consequently, the half-time of Polo dissociation from damaged chromatids was slower than that of BubR1 (Fig.
4H). These simultaneous dual-colour imaging data confirm our previous observations on the rapid association and slower dissociation kinetics of Polo at DNA breaks relative to BubR1.

**Polo is required for the robust recruitment of BubR1 and Bub3 to DNA lesions during mitosis**

The observation that Polo recruitment to DSBs precedes BubR1 and Bub3 prompted us to assess the requirement of Polo activity for the localization of BubR1 and Bub3 to sites of DNA damage. To do so, we monitored BubR1 and Bub3 dynamics in cells carrying a strong hypomorphic allele of *polo* (*polo*<sup>10</sup>)(Donaldson et al., 2001). Since attenuation of Polo activity induces a prolonged prometaphase (Conde et al., 2013; Llamazares et al., 1991; Sunkel and Glover, 1988), we compared the kinetics of BubR1 and Bub3 in *polo*<sup>10</sup> mutant cells with wild-type cells prolonged in prometaphase by colchicine treatment. Attenuation of Polo function severely altered the kinetics of BubR1 and Bub3 recruitment to DNA lesions (Fig. 5A-C). The initiation and half-time of recruitment of both BubR1 and Bub3 to DNA breaks were greatly delayed compared with their kinetics in colchicine-induced prometaphase-arrested cells (Fig. 5D-E). To confirm this result, we monitored the kinetics of GFP::BubR1 in wild type cells treated with the Polo inhibitor BI2536. Upon pharmacological inhibition of Polo activity, BubR1 signal was not detected at the site of DNA damage in the majority of cells (Fig. 5F). Collectively, these data indicate that Polo promotes the efficient recruitment of BubR1 and Bub3 to DNA breaks.

**The DNA damage sensors Mre11 and Rad50 transiently associate with DSBs during mitosis**

In a previous study, we reported that none of the core kinetochore proteins, including CenpA/Cid, CenpC, Spc105, Spc25, Nuf2, nor members of the SAC, Mps1, Mad1 or Mad2,
localized on I-Crel- or laser-induced DSBs (Derive et al., 2015; Royou et al., 2010). These data suggest that the formation of a neo-kinetochore on broken chromosomes is highly unlikely. Since Cid is required for the localization of Polo and BubR1 on kinetochores (Blower and Karpen, 2001), we reasoned that Polo and BubR1/Bub3 localization on DNA breaks is likely to be mediated via interactions with components that do not depend on kinetochore proteins. Recent studies in vertebrates reported that components of the early steps of the DDR, including the MRN complex, ATM and \( \gamma \)H2Ax, form foci on damaged chromosomes during mitosis (Giunta et al., 2010; Gomez-Godinez et al., 2010; Orthwein et al., 2014). Similarly, \( \gamma \)H2A.Z foci were detected on damaged chromatids in Drosophila neuroblasts (Royou et al., 2010), suggesting that the activation of the initial steps of the DDR in mitosis is conserved in Drosophila. It therefore seemed possible that the activation of the first steps of the DDR during mitosis promotes the recruitment of Polo and, consequently, BubR1 and Bub3 to DNA lesions.

Thus, we examined the dynamics of the DSB sensors, Mre11 and Rad50, on sites of laser-induced DNA damage in mitotic neuroblasts. One dose of mRFP-Rad50 rescued the lethality associated with a rad50\( ^{EP} \) strong hypomorph mutation, demonstrating that mRFP did not ablate Rad50 function. Next, the dynamics of GFP::Mre11 and mRFP::Rad50 were monitored on DNA lesions at different stages of mitosis. Mre11 and Rad50 signals appeared within 5 to 10 seconds following IR, which was even more rapid than Polo (Fig. 6A). As was the case for Polo, BubR1 and Bub3, Mre11 and Rad50 exhibited biphasic kinetics at DNA lesions with a phase of association promptly followed by a phase of dissociation (Fig. 6A, B). We also observed the re-association of Mre11 and Rad50 at sites of DNA damage coupled with their nuclear localization upon entry into the next interphase (Fig. 6A, last insets, B). Remarkably, the biphasic kinetics of Mre11 and Rad50 did not correlate with the timing of
anaphase onset, in contrast to Polo, BubR1, Bub3 and Fzy, (Fig. 6B). Consequently, the half-
time of Mre11 recruitment was less than one minute, regardless of the stage of mitosis at
which DSBs were induced (Fig. 6C). These observations suggest that the level of Cdk1 activity
has minimal effect on MRN kinetics at DSBs. However, when IR was induced during
anaphase, Mre11 did not reach the same levels on DSBs as observed during other points in
mitosis (Fig. 6D). This puzzling observation led us to test the influence of Cdk1 activity on
Mre11 dynamics at lesions by monitoring GFP::Mre11 in colchicine-treated, prometaphase-
arrested cells after IR. No difference in Mre11 kinetics was detected between cells going
through mitosis and prometaphase-arrested cells (Fig. 6C-E). This result suggests that, unlike
Polo, high Cdk1 activity does not prevent Mre11 dissociation from DSBs.

Next, we examined the differences in kinetics between the MRN complex and Polo
on mitotic DNA lesions by dual-color imaging of neuroblasts expressing both GFP::Mre11
and mCherry::Polo (Fig. 6F, G). As previously observed, GFP::Mre11 signal promptly
appeared at the site of IR, preceding mCherry::Polo (Fig. 6F-H). GFP::Mre11 recruitment to
DSBs was brief and it invariably initiated its dissociation in less than three minutes after IR,
regardless of the stage of mitosis (Fig. 6F, G). In contrast, mCherry::Polo remained associated
at the damaged chromosome consistently until anaphase onset (Fig. 6E, G, I). This point is
illustrated by the observation that its half-time of recruitment has a greater variance than
that of GFP::Mre11 (Fig. 6H).

**Depletion of MRN affects Polo kinetics at DNA lesions**

Since Mre11 precedes Polo at the site of IR, we hypothesized that the recognition of DSBs by
the MRN complex during mitosis may facilitate the recruitment of Polo and, subsequently,
BubR1 and Bub3 to DNA breaks. To test this idea, we monitored the dynamics of Polo in
**rad50** mutant cells. Rad50 is an essential gene in Drosophila, and null or strong hypomorphic alleles result in lethality at the pupal stage (Ciapponi et al., 2004; Gorski et al., 2004). Furthermore, Rad50 as well as Mre11 and Nbs1 have conserved functions in DNA repair signaling and telomere protection (Ciapponi et al., 2004; Ciapponi et al., 2006; Gorski et al., 2004; Sekelsky, 2017).

We first controlled that Mre11 was no longer detected on DSBs in mitosis in **rad50***EP* strong-hypomorph mutant cells (Fig. 7A). We then monitored the kinetics of recruitment of GFP::Polo in **rad50***EP* mutant cells after IR (Fig. 7B, C). We observed that attenuation of Rad50 severely altered Polo dynamics at DSBs. The time of Polo appearance at DNA lesions and its half-time of recruitment were both greatly delayed (Fig. 7D, E). Similarly, Polo did not reach the high levels at DNA lesions observed in wild-type cells in **rad50***EP* mutant cells (Fig. 7F). These results indicate that the MRN complex acts upstream of Polo at mitotic DSBs.

**Stable localization of Mre11 to chromatin is sufficient to recruit Polo independently of DNA damage**

Previous studies demonstrated that prolonged binding of the MRN complex to chromatin was sufficient to trigger the recruitment and activation of downstream components of the DDR independently of DNA damage (Bonilla et al., 2008; Soutoglou and Misteli, 2008). To determine whether ectopic localization of the MRN complex to chromatin was sufficient to recruit Polo independently of DNA lesions in mitosis, we fused Mre11 to the *E. coli* lactose repressor (LacI), tagged with GFP. The construct was introduced into flies carrying an X chromosome with 256 repeats of the lac operator sequence (LacO) located near the telomere (Fig. 8A) (Belmont and Straight, 1998). We first controlled that the fusion protein was efficiently recruited to laser-induced DNA damage during mitosis, and that it co-
localized with mRFP::Rad50 and mCherry::Polo at the site of damage (Fig. S4a, B). Next, we monitored the localization of the fusion proteins in flies carrying one X chromosome with 256 lacO repeats. As expected, GFP::LacI::Mre11 signal was observed as distinct foci on lacO arrays in 73% of mitotic cells (Fig. 8B). We did not observe any obvious defects in chromatid segregation, suggesting that the persistent tethering of Mre11 to chromatin did not affect genome integrity. In the seven mitotic cells where GFP::LacI::Mre11 was detected on LacO arrays, three cells exhibited mRFP::Rad50 foci co-localizing transiently with GFP::LacI::Mre11. This result indicates that the ectopic localization of Mre11 on chromatin resulted in the recruitment of Rad50, as demonstrated in human cells (Fig. 8B) (Bonilla et al., 2008; Soutoglou and Misteli, 2008). Remarkably, mCherry::Polo signal was also detected with GFP::LacI::Mre11 foci in 25% of cells (Fig. 8B). This result indicates that the tethering of Mre11 to chromatin is sufficient to recruit Polo independently of DNA damage.

The DNA repair component Okra does not localize on mitotic DSBs.

In vertebrates, the pathway downstream of MRN and ATM involved in DNA repair by NHEJ or HR is inhibited during mitosis (Giunta et al., 2010; Orthwein et al., 2014; Peterson et al., 2011; Terasawa et al., 2014). To determine if this inhibition is conserved in Drosophila, we monitored the dynamics of Okra after IR (Drosophila homolog of Rad54), a helicase involved in DNA repair by HR (Kooistra et al., 1999; Schupbach and Wieschaus, 1991; Sekelsky, 2017). As expected, a prominent Okra::GFP focus was detected at the site of laser-induced damage in interphase nuclei, indicating that DNA repair by HR was initiated (Fig. S5, cell#2). In contrast, no Okra::GFP signal was observed on damaged chromosomes in mitotic cells (Fig. S5, Cell#1). Collectively, theses results provide evidence that, similar to vertebrates, the
early steps of the DDR is activated upon DNA damage in Drosophila mitotic cells. This is followed by inhibition of the Okra-dependent downstream repair pathway.

Discussion

Using live single-cell analysis combined with surgical damage of one chromosome at a defined time during mitosis, we have performed detailed analysis of the kinetics of the mitotic proteins Polo, BubR1, Bub3 and Fzy and the DSB sensors Mre11 and Rad50 on mitotic DSBs. Our studies show that Mre11 and Rad50 transiently associate with DSBs and facilitate the robust recruitment of Polo and, consequently, BubR1/Bub3 to DSBs. This, combined with our previous observation that Polo/BubR1/Bub3 are required for the tethering of the two broken chromosome ends can be integrated within the following model: DSBs in mitosis are promptly marked by the MRN complex, promoting the recruitment of Polo kinase (Fig. 8C). Subsequently, the MRN complex dissociates from DSBs, possibly to prevent the activation of downstream repair components, while Polo kinase activity promotes the subsequent accumulation of the BubR1/Bub3 complex at DSBs. This complex sequesters Fzy, thereby inhibiting the APC/C locally. Polo and BubR1/Bub3 levels are maintained at DSBs until mid-anaphase, thus facilitating the persistent tethering of the broken fragments at a critical time during poleward chromosome movement. Upon segregation of the broken DNA fragment, Polo and the BubR1/Bub3/Fzy complex dissociate from the DNA lesions. However, a pool of Polo persists on DSBs through the next interphase, while MRN re-accumulates at the site of damage to promote downstream repair signaling (Fig. 8C)(Derive et al., 2015; Royou et al., 2010).
**Polo kinetics at mitotic DSBs.**

We observe that Polo promptly accumulates at DSBs at any point during mitosis, including anaphase, albeit with lower levels. The kinetics of its recruitment are sigmoidal, suggesting the cooperative binding of Polo at the site of damage. Given that Polo localization to sub-cellular structures is mediated through its interaction with primed phosphorylated substrates (Elia et al., 2003a; Elia et al., 2003b; Schmucker and Sumara, 2014), our findings that pharmacological inhibition of Polo kinase activity severely alters Polo recruitment to DSBs, but does not completely prevent it, supports the conclusion that Polo kinase activity is not required for the initial steps of DSB association but promotes its cooperative binding to DSBs through a self-priming mechanism. Interestingly, recent studies have reported that Mre11 is a substrate of Plk1 in human cells (Li et al., 2017; Xu et al., 2018). It is therefore conceivable that Polo promotes its recruitment to DSBs via phosphorylation-dependent priming of Mre11.

Several lines of evidence suggest that Cdk1 activity modulates Polo kinetics at DSBs. First, Polo initiates its dissociation from DSBs invariably during anaphase, in concert with the decline of Cdk1 activity. Second, Polo levels are constantly maintained at DSBs in prolonged prometaphase when Cdk1 activity is high. Finally, Polo never reaches the high levels on DSBs created during anaphase. While the molecular mechanism by which Cdk1 contributes to Polo kinetics at DSBs remains to be elucidated, we can propose plausible possibilities. Numerous Polo substrates are primed by Cdk1 (Lowery et al., 2005); thus, Cdk1 may facilitate the enrichment of Polo at DNA lesions by priming Polo targets associated with DSBs. Alternatively, Cdk1 may sustain Polo activation at DSBs directly through the
phosphorylation of the T-loop in Polo kinase domain (Mortensen et al., 2005), or indirectly by inhibiting counteracting phosphatases. The observation that Polo is successfully recruited to DSBs induced at anaphase, albeit with less efficiency, suggests that Cdk1 activity is not essential for the initial steps of Polo recruitment to DSBs but is important for its cooperative binding and its maintenance to the lesions.

**Polo promotes the robust recruitment of BubR1/Bub3 to mitotic DSBs.**

In this study we demonstrate that one of Polo’s functions at mitotic DSBs is to target the BubR1/Bub3 complex. Polo precedes BubR1/Bub3 on DSBs and attenuation of its kinase activity dramatically altered the kinetics of BubR1/Bub3 at DSBs. This raises the question of how Polo mediates the recruitment of BubR1/Bub3 to DSBs. One possibility is via direct phosphorylation of BubR1 by Polo. In a previous study, we found that a 432 amino acid fragment of BubR1 (from amino acid 330 to amino acid 762) was sufficient for its localisation to mitotic DSBs. This sequence encompasses a conserved motif called KARD, which is phosphorylated by Plk1 in human cells, resulting in the interaction of BubR1 with the phosphatase PP2A-B56α at kinetochores (Espert et al., 2014; Kruse et al., 2013; Suijkerbuijk et al., 2012). However, it is unlikely that Polo mediates the localization of BubR1/Bub3 to DSBs via direct phosphorylation of the BubR1 KARD motif, since mutation of the putative Polo phosphosites to alanine or aspartate had no obvious effect on the localization of BubR1 to mitotic DSBs (Derive et al., 2015). However, this 432aa sequence includes 7 other Polo putative phosphosites, including serine at position 581, which is phosphorylated in vivo in Drosophila embryos (Zhai et al., 2008). It would be relevant to determine if any of these putative Polo phosphosites are important for BubR1 targeting to DSBs. Alternatively, Polo may target BubR1/Bub3 to DSBs indirectly by promoting interactions with another substrate.
Studies have shown that Bub3 is targeted to the kinetochore via direct interaction with the kinetochore scaffold KNL1. This interaction involves the recognition of phosphorylated MELT repeats in KNL1 by two residues in Bub3 (Primorac et al., 2013). Importantly, although the MELT repeats are phosphorylated primarily by the spindle checkpoint component Mps1, studies have shown that Plk1 also contributes to this phosphorylation (Espeut et al., 2015). Interestingly, sequence analysis reveals that Drosophila Rad50 contains two MELT repeats. In addition, the residues identified in human Bub3 that are critical for its interaction with the phosphorylated MELTs are conserved in Drosophila. Although speculative, it is possible that Polo phosphorylates Rad50 on its MELT repeats at mitotic DSBs, and these phospho-MELTs, in turn, target the BubR1/Bub3 complex via direct interaction with Bub3. It would be relevant to determine the importance of the conserved Bub3 residues and the Rad50 MELT repeats in the recruitment of BubR1/Bub3 to DSBs.

**MRN associates transiently to mitotic DSBs.**

While the detection of DSBs in interphase may be facilitated by the confinement of the DSBs sensors in the nucleus and their association to chromatin, the recognition of DSBs during mitosis poses a greater challenge, as the DSBs sensors are dispersed throughout the cytoplasm (Polo and Jackson, 2011). Here, we show that cytoplasmic Mre11 and Rad50 readily accumulate on DSBs in mitosis with a half-time of recruitment estimated at around 30-40 seconds, modestly faster than that observed for human Rad50 on interphase DSBs using the same method of micro-irradiation \((t_{1/2} \sim 60\text{sec})\) (Aleksandrov et al., 2018). This is consistent with the detection of mitotic and interphase DSBs by MRN via a conserved mechanism. Experiments using single molecule tracking of MRN on *in vitro* reconstituted DNA homoduplexes have provided evidence that chromatin-associated MRN detects free
DNA ends by scanning the DNA molecule through facilitated diffusion (Myler et al., 2017). However, since MRN is excluded from the condensed chromosomes, this chromatin-dependent diffusion-based mechanism is unlikely to occur on mitotic DSBs. It may be that the initial detection of free DNA ends occurs through a different pathway, possibly through cytoplasmic diffusion but, once associated, a minimal amount of MRN triggers the rapid and robust accumulation of additional MRN molecules via cooperative binding, as suggested by the sigmoidal shape of Mre11 recruitment to mitotic DSBs. We cannot rule out that an undetected pool of MRN complex remains associated to condensed chromatin during mitosis and senses DSBs through active diffusion as demonstrated in vitro (Myler et al., 2017).

The recruitment of Mre11 and Rad50 to mitotic DSBs is strikingly transient. Maximum levels are observed ~2min following laser irradiation and they subsequently dissociate rapidly from DSBs ($t_{1/2}$ dissociation ~2min), until the next interphase where they re-accumulate on the lesions. Although, the kinetics of Drosophila MRN at DSB during interphase is not known, our result contrasts with observations of human Rad50 in the interphase nucleus, where its removal from DSBs is slow ($t_{1/2}$$>$30min)(Aleksandrov et al., 2018). We currently do not know the biological significance of the prompt dissociation of Rad50 and Mre11 from mitotic DSBs and how it is controlled. One possibility is that the transient association of MRN with mitotic DSBs is sufficient to initiate the recruitment of Polo and, consequently, BubR1/Bub3, but its ensuing rapid dissociation prevents the unscheduled recruitment of downstream DNA repair components to damage chromatin, which could otherwise compromise sister chromatid segregation. This idea is consistent with the observations that DNA repair signaling downstream of MRN is inhibited during mitosis (Giunta et al., 2010; Orthwein et al., 2014; Peterson et al., 2011; Terasawa et al., 2014)(this
study) and that its reactivation provokes telomere fusion (Orthwein et al., 2014). If this model is correct, one might expect to observe an alteration of chromosome structure that would lead to segregation defects upon prolonged association of MRN with mitotic DSBs. However, no noticeable defects in chromosome segregation were observed in cells where Mre11 was persistently tethered to X chromosome-associated lacO arrays. These results should be interpreted cautiously since mRFP::Rad50 co-localized with lacO-associated GFP::LacI::Mre11 and mRFP::Rad50 signal was associated with large cytoplasmic aggregates. Therefore, insufficient functional MRN complex may be generated using this approach to induce a dominant negative effect. The future challenge will be to decipher the underlying mechanisms that control MRN kinetics on mitotic DSBs.

**MRN promotes the robust recruitment of Polo to mitotic DSBs**

We found that depletion of MRN severely alters Polo kinetics at mitotic DSBs. Conversely, ectopic targeting of Mre11 to chromosomes is sufficient to recruit Polo, albeit with low efficiency. Collectively, these data strongly argue that the MRN complex plays a positive role in targeting Polo to DSBs. As discussed above, MRN might promote Polo association with DSBs via direct interaction with Mre11. Alternatively, MRN might target Polo to DSBs indirectly through the recruitment of additional DNA repair components that interact with Polo. In interphase, MRN recruits several effectors including MDC1 and C-terminal Binding Protein Interacting Protein (CtIP) to DSBs. Both MDC1 and CtIP are also found on mitotic DSBs (Giunta et al., 2010; Leimbacher et al., 2019; Wang et al., 2018). In addition, MDC1 interacts with TopBP1 on damaged chromosomes where they form filaments reminiscent of the Polo-coated tethers observed in Drosophila (Leimbacher et al., 2019; Royou et al., 2010).
Importantly, systematic human interactome studies revealed an interaction between Plk1 and TopBP1 (Huttlin et al., 2015). Finally, recent studies reported that the presence of DSBs in nocodazole-treated human cells triggers the Plk1-dependent phosphorylation of CtIP (Li et al., 2017). Therefore, it is possible that the MRN complex mediates the recruitment of Polo to DSBs indirectly by promoting the accumulation of one of its substrates such as TopBP1 or CtIP. The fact that Polo is maintained at DSBs until mid-anaphase or in prolonged prometaphase, at a time MRN has already dissociated from DSBs, supports the notion that MRN is required for the initiation of Polo recruitment to mitotic DSBs, but, unlike Cdk1, is dispensable for its maintenance.

Future studies will determine the molecular mechanisms by which MRN and Cdk1 control Polo association with mitotic DSBs and how Polo, in turn, facilitates the recruitment of BubR1/Bub3 to broken chromosomes. Current anti-cancer therapies rely heavily on the induction of extensive DNA damage, either by irradiation or genotoxic agents. Thus, deciphering the molecular pathways that process DNA damage in healthy mitotic cells will improve our understanding of tumor resistance to these therapies and may help identify novel targets that will enhance the efficacy of these therapies.
Methods

Fly strains

Flies were grown on corn flour and yeast extract medium under standard conditions at 25°C. The h2az>H2A.Z::mRFP stock was obtained from the Bloomington Drosophila Stock Center (Indiana University, USA). bub3>EGFP::Bub3 stock was provided by C. Lehner (Institute of Molecular Sciences, University of Zurich, Switzerland). The bubR1>GFP::BubR1 stock was provided by R. Karess (Institute Jacques Monod, Paris, France). The polo>GFP::Polo stock was provided by C. Sunkel (Instituto de Biologia Molecular e Celular, Porto, Portugal). The ubi>GFP::Fzy stock was provided by J. Raff (Sir William Dunn School of Pathology, University of Oxford, UK). The GFP::Jupiter are described in (Karpova et al., 2006). The bubR1[1] (Basu et al., 1999), polo[10] (Donaldson et al., 2001) and rad50[EP1] (Ciapponi et al., 2004; Gorski et al., 2004) mutations were previously described. The transgenic stocks ubi>GFP::Mre11, ubi>GFP::LacI::Mre11, ubi>mRFP::Rad50, ubi>Okra::GFP and polo>mCherry::Polo were produced in our laboratory. The transgenic stock ubi>GFP::Mre11 and ubi>mRFP::Rad50 are described in (Murcia et al., 2019).

Molecular cloning

All plasmids were verified by sequencing before being injected into fly embryos to generate transgenic stocks (Bestgene Inc.).

ubi>GFP::LacI::Mre11 cloning: The LacI sequence was amplified from the pGEX6p2 vector using primers that added the AttB1 and AttB2 Gateway sequence and the sequence of a
GAGAGAGA flexible peptide linker to the N and C-termini of LacI respectively. The Mre11 coding sequence was amplified from pENTR-N-Mre11 vector with primers that added the GAGAGAGA linker and AttB1 and AttB2 gateway sequence on N and C-termini of Mre11 respectively. The two PCR products were combined and amplified to get the final PCR product that was inserted into the entry gateway vector. This product was recombined with a pUbi-GFP::GAT destination vector (a gift from J. Raff) to obtain the plasmid ubi>GFP::LacI::Mre11.

**ubi>Okra::GFP cloning:** The Okra coding sequence was amplified from pOT2-Okr vector (DGRC, plasmid n°LD35220) using primers that added the AttB1 and AttB2 Gateway sequences on the N and C termini of Okra respectively. The PCR product was inserted into the pDONR/Zeo vector (Life technologies). This resulting entry vector was recombined into the pUbi-GAT::GFP vector containing the ubiquitin promoter and a C-terminal GFP tag (a gift from J. Raff).

**polo>mCherry::Polo cloning:** The polo>mCherry::Polo construct was obtained by multistep cloning. First, a region of 881 nucleotides upstream of the polo initiation codon was amplified from genomic DNA using primers introducing BamHI and EcoRI restriction sites. mCherry was amplified with a forward primer introducing an EcoRI restriction site and a reverse primer introducing a NotI site plus a GAGAGAGA linker. The cDNA Polo sequence was amplified with foward and reverse primers introducing NotI and Kpnl sites respectively. A region of 704 nucleotides after the STOP codon of PoloCDS was amplified from genomic DNA using forward and reverse primers introducing XhoI and KpnI sites respectively. Each PCR product was first inserted into a pGEM-T easy vector. After digestion, the products were
ligated into a pAttB vector. Ligation of this product resulted in the final pAttB polo>mCherry::Polo vector.

**Live analysis of larval Neuroblasts**

For live imaging of neuroblasts, late third-instar larvae were dissected in PBS (phosphate buffer saline, 1X) and their central nervous system transferred in a drop of PBS on a coverslip. The brain was gently compressed by capillary action and the coverslip was sealed with Halocarbon oil 700 (sigma). Each preparation was monitored for less than one hour. For colchicine and BI2536 treatments, whole brains were incubated in PBS containing 10 µM Colchicine (Sigma) and/or 1 µM BI2536 (Selleckchem) for 30 minutes before preparation for live imaging as described above. These conditions of colchicine and BI2536 treatment were optimal to produce >30min delay in prometaphase. Live analysis was performed at room temperature with a 100x oil Plan-Apochromat objective lens (NA 1.4) and an Axio-Observed.Z1 microscope (Carl Zeiss) equipped with a spinning disk confocal (Yokogawa), an EMCCD Evolve camera (Photometrics and Roper Scientific), 491 nm (100 mW; Cobolt calypso) and 561 nm (100 mW; Cobolt Jive) lasers. Images were acquired with Metamorph software (Roper Scientific). 12 Z-series of 0.5 µm steps were acquired every 5 and/or 20 seconds. All images are maximum-intensity projections. 355 nm microchip laser (Teem Photonics) (passively Q-switched SNV-20F-000) with a 21 kHz repetition rate, 0.8 µJ energy/pulse, 2 kW of peak power and 400 ps pulse width, powered with an iLas2 PULSE system (Roper Scientific) was used at 10, 15, 20 or 30% power with one pulse of a spot length of 100 points to induce surgical damage to one chromosome. 20% laser power was used for all experiments unless specified.
Data analysis and quantification

ImageJ (Fiji, National Institute of Health) was used for image analysis and fluorescence quantification. The level of our fluorescent-tagged proteins at DNA lesions was measured as followed: the average fluorescence intensity of a defined region of interest at the site of IR (ROI A) and the average fluorescence intensity of the cytoplasm near the site of IR (ROI B) were measured over time from maximum projection images (12z of 0.5 µm depth). The background of the camera was removed for each value. For each time point, the level of signal at the site of damage was equal to the difference between the average signal of ROI A and B divided by the average signal in ROI B ([(ROIA-background)-(ROIB-background)]/(ROIB-background)). The initiation of recruitment at DNA lesions corresponds to the time where the difference between ROI A and B signals is positive. To determine the half-time of recruitment ($t_{1/2}$) and maximum level parameters, each data set (from 0min to the time of peak level) was fitted to the following sigmoid function: $Y=\text{Max}/[1+10^{([(t_{1/2})-X)\ast\text{Hillslope}]}]$ using prism (Graphpad) where Max=maximum level. To estimate the half-time of dissociation, each data set was normalized to max=1 and the data from $Y=1$ to bottom plateau was fitted to the following sigmoid function: $Y=\text{Bottom}+(1-\text{Bottom})/[1+10^{([(t_{1/2})-X)\ast\text{Hillslope}]}]$. In Fig. 1H, 4B, 4C and 6G, the data were normalized to max=1.
Acknowledgments

We thank Derek McCusker and Cameron Mackereth, as well as Royou team members for fruitful discussion and critical reading of the manuscript. This work was supported by the Centre National de la Recherche Scientifique (CNRS)(E.M., A.R.), the Conseil Régional d’Aquitaine (20111301010)(C.L, A.R.) the university of Bordeaux (M.C.C.) and the European Research Council (GA311358-NoAneuploidy)(G.G.D., P.P.E and A.R.).

Author contributions

PPE, CL, DGG, EM, MCC and AR performed the experiments. AR supervised the work and wrote the manuscript.

Competing interests: the authors declare that no competitive interests exist.
References


Figure 1. Polo is recruited to laser-induced DNA breaks in mitosis and its maintenance at DNA lesions is coupled with Cdk1 activity. (A) Time-lapse images of Drosophila third instar larvae neuroblasts expressing H2Az::mRFP (red) and GFP::Polo (cyan) after micro-irradiation (IR) of one chromosome during prometaphase (top panels), metaphase (second panels), anaphase (third panels) and telophase/G1 phase (bottom panels). Yellow arrowheads
indicate the site of IR. The dashed squares represent the position of the insets. Time=min:sec. Time=0:00 corresponds to the time of IR. The white arrows point to the localization of the GFP::Polo signal at IR-induced DNA lesions. The insets show the inverted GFP::Polo signal at the site of IR. The % corresponds to the frequency of cells with a detectable GFP::Polo signal at the site of IR. Scale Bar=5 µm and 1 µm for panels and insets, respectively. (B) Graphs showing one example of GFP::Polo levels over time at DNA lesions after IR (dashed lines) induced during prometaphase (PM), metaphase (M) or anaphase (A). The time is relative to anaphase onset (t=0). (C, D, E, F) Scatter dot-plots showing the time of appearance (C), half-time of maximum recruitment (t_{1/2})(D), onset of dissociation (E) and maximum levels (F) of GFP::Polo at the site of damage for the indicated conditions. (G) Time-lapse images of a neuroblast expressing H2Av::RFP (red) and GFP::Polo (cyan) arrested in prometaphase after 30 minutes treatment with colchicine (10 µM). The yellow arrowhead indicates the site of damage and the white arrow indicates the accumulation of GFP::Polo signal at the site of damage. Scale Bar=5 µm. Time=min:sec. (H) Graph displaying the average kinetics of GFP::Polo at DNA lesions in untreated (Untr) cells (which correspond to cells where the lesions were created in PM and that have been monitored for more than 12 min) and in colchicine (Colch) treated cells. The dots and bars correspond to mean±95%CI. The yellow line corresponds to the data fit to a sigmoid equation \( Y = \text{Max}/\left(1 + 10^{\left[\left(\text{t}_{1/2}-X\right)\times\text{Hillslope}\right]}\right) \) and the black line corresponds to the data smoothed with 4 averaged neighbors. n=number of cells. (I, J) Scatter dot-plots showing the half-time of recruitment (t_{1/2})(I) and maximum levels (J) of GFP::Polo at the site of damage in untreated cells (Untr, as shown in D for PM cells) and cells treated with colchicine (Colch). Lines and bars for the scatter dot-plots correspond to median±interquartile range. Mann-Whitney two-tailed tests were used to calculate P values.
Figure 2. Inhibition of Polo kinase activity with BI2536 alters Polo kinetics at DSBs in mitosis. (A) Time-lapse images of neuroblasts expressing H2Az::mRFP (red) and GFP:Polo (cyan) after IR (yellow arrowhead, t=0) previously treated with colchicine (top panels), BI2536 (middle panels) or both colchicine and BI2536 (bottom panels) for 30 minutes. The white arrows indicate the localization of the proteins on DNA breaks. Insets show GFP::Polo inverted signal at the site of damage. Time=min:sec. Scale Bar=5 µm and 1 µm for images and insets, respectively. (B) Graph displaying the average kinetics of GFP::Polo at DNA
lesions in cells pre-treated with colchicine (Colch), BI2536 or both. Dots and bars represent mean±95%CI. The data were fit to the sigmoid equation: \[ Y=\text{Max}/\{1+10^{\left[\left(t_{1/2}-X\right)\times\text{Hillslope}\right]}\} \]

n=number of cells. (C) Scatter dot-plot showing the time when GFP::Polo is initially detected at laser-induced DNA lesions in cells pre-treated with colchicine (Colch), BI2536 or both. (D) Scatter dot-plot displaying the half-time of recruitment (t_{1/2}) of GFP::Polo at the site of IR in cells treated with colchicine, BI2536 or both. (E) Scatter dot-plot displaying the maximum levels of GFP::Polo on DSBs in cells treated with colchicine (Colch), BI2536 (Bi) or both. The lines correspond to median ±interquartile range. A Mann-Whitney two-tailed test was used to calculate P values.
Figure 3. BubR1, Bub3 and Fzy exhibit similar biphasic kinetics at DNA breaks in mitosis. (A) Time-lapse images of neuroblasts expressing H2Az::mRFP (red) and the indicated GFP-tagged proteins after IR induced during prometaphase (PM), metaphase (M) or anaphase (A). The yellow arrowheads indicate the site of damage. The white arrows indicate the localization of the proteins on DNA breaks. The frequency (%) of cells with a detectable signal of the indicated protein at DNA lesions as well as the total number of cells monitored (n) is specified under each panel. Time=min:sec. Scale Bar=5 µm. (B) Graphs representing one example of kinetics of the indicated protein on DNA lesions depending on the stage of mitosis at which IR was induced (dashed line). The time 0 corresponds to anaphase onset. (C, D, E) Scatter dot-plots showing the time when the indicated protein is initially detected at...
DNA lesions (C), the half-time of recruitment ($t_{1/2}$)(D) and the onset of dissociation of the indicated proteins from the lesions (E). The red bars represent median ± interquartile range. The three proteins display similar kinetics on DSBs, consistent with their localization as a complex. No significant difference between the kinetics of the three proteins was observed using a Mann-Whitney two-tailed test.
Figure 4. BubR1 is recruited after Polo to DNA lesions and, unlike Polo, completely dissociates from the lesions at the end of mitosis. (A) Time-lapse images of neuroblasts expressing GFP::BubR1 (grey in top panels and insets, cyan in bottom panels) and mCherry::Polo (grey in middle panels and insets, red in bottom panels). The yellow arrowheads indicate the site of damage. The cyan and red arrows indicate the localization of GFP::BubR1 and mCherry::Polo on DNA breaks. The insets in the top and middle panels are a magnification of the region of IR. Time=min:sec. Scale Bar=5 µm and 1 µm for images and insets respectively. (B) Graphs displaying the kinetics of the indicated proteins on DNA breaks.
lesions in one cell. The data are normalized so that the maximum levels for each protein equal 1. The lines correspond to the data smoothed with 4 averaged neighbors. The dashed line indicates the time of anaphase onset. Time 0 corresponds to the time of IR. (C) Graphs displaying the average kinetics of mCherry::Polo and GFP::BubR1. The lines correspond to the data smoothed with 4 averaged neighbors. Bars=95% CI. n= number of cells quantified. The dashed line represents the average time of anaphase onset. (D, E) Scatter dot-plots displaying the initiation (D) and half-time ($t_{1/2}$)(E) of recruitment of the indicated proteins to DNA lesions. (F, G, H) Scatter dot-plots displaying the onset (F), half-time (G) and complete (H) dissociation of mCherry::Polo and GFP::BubR1 from the lesions after anaphase onset (AO). The lines and bars represent median ±interquartile range. A Mann-Whitney two-tailed test was used to calculate P values.
Figure 5. Attenuation of Polo activity affects BubR1 and Bub3 kinetics at DNA lesions in mitosis. (A) Time-lapse images of neuroblasts expressing H2Az::mRFP (red) and GFP::BubR1 (cyan in top panels, grey in insets) or GFP::Bub3 (cyan in bottom panels and grey in insets) after IR (yellow arrowheads, time=0). The kinetics of GFP::BubR1 and GFP::Bub3 at DNA lesions after IR shows differences in their localization and interaction with other proteins.
lesions were compared between wild type cells arrested in prometaphase after colchicine treatment (Colch) and polo10 mutant cells. The white arrows indicate the localization of the proteins on DNA breaks. The dashed squares represent the inset regions. (B and C) Kinetics of GFP::BubR1 (B) and GFP::Bub3 (C) at DNA breaks (DSBs) after IR (t=0). Dots and bars correspond to mean±95%CI. The data were fit to the following sigmoid function: \[ Y = \text{Max}/\{1+10^\left[\left((t_{1/2})-X\right)\times\text{Hillslope}\right]\} \]. (D, E) Scatter dot-plot showing the initiation (d) and half-time (t_{1/2}) (E) of recruitment of the indicated proteins to DSBs in colchicine-treated WT cells and polo10 mutant cells. The bars correspond to median ±interquartile range. A Mann-Whitney two-tailed test was used to calculate P values. (F) Time-lapse images of neuroblasts expressing GFP::BubR1 (cyan and insets) and H2Av::mRFP (red) treated with the Polo inhibitor BI2536 after IR (yellow arrowhead, t=0). Top panels show one of 3 out of 17 BI2536-treated cells where GFP::BubR1 signal is detected on DSBs (white arrow, insets). Bottom panels display one of 14 out of 17 BI2536-treated cells where no GFP::BubR1 signal is detected on DSBs. Scale Bar=5 µm and 1 µm for images and insets, respectively. The dashed square represents the inset region. Time=min:sec. A Mann-Whitney two-tailed test was used to calculate P values.
Figure 6. Mre11 and Rad50 precede Polo on IR-induced DSBs but, unlike Polo, dissociate rapidly from the lesions regardless of the stage of mitosis. (A) Time-lapse images of neuroblasts expressing H2Az::mRFP (red) and GFP::Mre11 (top panels, cyan and insets) or H2Az::GFP (red) and mRFP::Rad50 (bottom panels, cyan and insets) after IR (yellow arrowhead, t=0). The white arrows highlight the localization of the indicated proteins on DNA lesions. Time: min:sec. Scale bars are 5 µm and 1 µm for images and insets.
respectively. (B) Graphs showing representative kinetics of GFP::Mre11 at DNA lesions after IR induced during prometaphase (PM), metaphase (M) or anaphase (A). Time 0 corresponds to anaphase onset (AO). (C) Graphs showing the kinetics of GFP::Mre11 at DNA lesions after IR in untreated cells (untr, where IR was induced during prometaphase) or cells previously treated with colchicine (Colch). Dots and bars represent mean ± 95%CI. The lines correspond to the data smoothed with four averaged neighbors. (D, E) Scatter dot-plots showing the half-time of recruitment (t_{1/2}) (D) and the maximum levels (E) of GFP::Mre11 for the indicated conditions. Time 0 corresponds to the time of IR. The bars correspond to median ± interquartile range. (F) Images of neuroblasts co-expressing mCherry::Polo (red) and GFP::Mre11 (Cyan). IR was induced at prometaphase (cell1, top panels) and metaphase (cell2, bottom panels). The yellow arrowheads point to the site of IR. The following insets correspond to the inverted mCherry::Polo (top row) and GFP::Mre11 (bottom row) channels at the site of IR (delineated by a dashed square in the first images). The frames corresponding to the time of anaphase onset is highlighted with a gray square for each cell. (G) Graphs showing the corresponding kinetics of GFP::Mre11 and mCherry::Polo at DNA lesions for cell 1 (solid line) and cell 2 (dashed line) shown in (A). (H, I) Scatter dot-plots showing the half-time of recruitment (t_{1/2}) (H) and onset of dissociation (I) of GFP::Mre11 and mCherry::Polo from the DNA lesions. The bars correspond to median ± interquartile range. A Mann-Whitney two-tailed test was used to calculate P values.
Figure 7. The robust recruitment of Polo to DNA breaks in mitosis depends on Rad50. (A) Time-lapse images of rad50$^{EP}$ mutant neuroblasts expressing H2Az::mRFP (red) and GFP::Mre11 (gray in top panels and cyan in bottom merged panels) after IR (yellow arrowhead, t=0). No GFP::Mre11 signal is detected on DNA breaks in 100% of rad50$^{EP}$ mutant cells (n=15). The dashed lines delineate the cell. Time: min:sec. Scale bar=5 µm. (B) Time-lapse images of wild-type (WT, top panel) and rad50$^{EP}$ mutant (bottom panel) neuroblasts expressing H2Az::mRFP (red) and GFP::Polo (cyan and insets) after IR (yellow arrowheads, t=0). The white arrow indicates the localization of GFP::Polo at the site of damage. * corresponds to the time of appearance of GFP::Polo on DSBs. Insets correspond...
to the GFP::Polo level at the site of damage (dashed squares on the first images). Dashed lines delineate the cells. Time: min:sec. Scale bar=5 µm and 1 µm for images and insets. (C) Graph displaying the average kinetics of GFP::Polo at the site of damage in wild type and rad50^{EP} mutant cells after IR. Dots and bars correspond to mean±CI95%. The data are fit to the following sigmoid equation: Y=Max/[1+10^{[(t_{1/2})-X]*Hillslope}]. (D, E, F) Scatter dot-plots showing the initiation (D), half-time (t_{1/2}) of recruitment (E) and maximum level of GFP::Polo at the site of damage for the indicated genotype. The lines and bars correspond to median ±interquartile range. A Mann-Whitney two-tailed test was used to calculate P values.
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#### MITOSIS

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#### INTERPHASE

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**Legend:**
- DNA damage response
- MRN
- Polo
- BubR1/Bub3/Fzy
- Active APC/C
- DNA repair signaling
Figure 8. Ectopic localization of Mre11 on chromatin is sufficient to recruit Polo independently of DNA damage. (A) Scheme of Drosophila chromosome X containing 256 LacO repeats located at position 1E. These repeats are binding sites for the E. Coli transcriptional repressor LacI. The expression of GFP::LacI fused to Mre11 suffices to target Mre11 to the LacO repeats. (B) Still images from time-lapse movies of neuroblasts expressing GFP::LacI::Mre11 (top panels and insets, red in merge) and H2Az::mRFP (mid panels and cyan in merge), mRFP::Rad50 (mid panel and inset, cyan in merge) or mCherry::Polo (mid panel and inset, cyan in merge). Time: min:sec. Scale Bar=5 µm and 1 µm for images and insets respectively. The red arrows indicate the GFP::LacI::Mre11 lacO arrays. The corresponding % of cells positive for GFP::LacI::Mre11 signal on lacO arrays and % of cells with mRFP::Rad50 or mCherry::Polo signals co-localizing with GFP::LacI::Mre11 on lacO arrays. n=number of cells. (C) Model for the DNA damage response in mitosis. After DNA damage, the MRN complex is promptly but transiently recruited to DSBs and targets Polo to the site of damage. Polo subsequently promotes the recruitment of the BubR1/Bub3 complex, which sequesters Fzy and inhibits the APC/C at the site of DNA damage during poleward movement of sister chromatids. BubR1/Bub3 dissociate from the DSBs at telophase, while a pool of Polo remains associated with DSBs into the next interphase. Meanwhile, MRN re-accumulates on DSBs, promoting the activation of downstream repair components.
Supplementary data

Supplementary figure 1

A

B

C

D

E
**Figure S1. GFP::Polo kinetics at DSB induced by a Pulsed UV laser used at different power percentages during mitosis.** (A) Time-lapse images of neuroblasts expressing H2Az::mRFP (red) and GFP::Polo (cyan) before and after micro-irradiation (IR) of one chromosome with a 355 nm pulsed laser at 15% power. Yellow arrowheads indicate the site of IR. Time=min:sec. Time=0:00 corresponds to the time of IR. The top panels show a cell with no detectable GFP::Polo signal at the site of damage. The bottom panels show a cell where GFP::Polo signal is detected at the IR-induced DNA lesions (the white arrows and insets). The white dashed square represents the inset region. The insets show GFP::Polo inverted signal at the site of damage. Scale Bar=5 µm and 1 µm for panels and insets, respectively. (B) Graph showing the frequency of cells with a detectable GFP::Polo signal at the site of IR for the indicated conditions. (C, D) Scatter dot plots showing the time of appearance (C) and the maximum levels (D) of GFP::Polo for the indicated conditions. Lines represent median ± interquartile.

(E) Time-lapse images of a neuroblast expressing H2Az::mRFP (red) and GFP::Polo (cyan) after micro-irradiation (IR, yellow arrowheads) of define zone in the cytoplasm. Time=min:sec. Time=0:00 corresponds to the time of IR. Scale Bar=5µm.
Supplementary figure 2

Figure S2. GFP::Polo kinetics at DSB induced at different stages of mitosis. (A) Graph showing the distribution of the $t_{1/2}$ of GFP::Polo recruitment to DSBs with the time of IR relative to anaphase onset (AO). A linear regression shows a correlation between the $t_{1/2}$ and the time of IR relative to AO. (B) Graph showing the distribution of GFP::Polo maximum levels at DSBs with the time of IR relative to anaphase onset (AO). A linear regression shows no correlation between maximum levels and the time elapsed between IR and AO. Lines correspond to mean±SD.
**Supplementary figure 3**

**Figure S3.** Complete depolymerisation of microtubules in mitotic neuroblasts upon **colchicine treatment.** Time-lapse images of neuroblasts expressing H2Az::mRFP (red) and the microtubule-binding protein GFP::Jupiter (cyan) after 30min incubation with PBS alone (untreated, top panels) or PBS plus 10µm colchicine (colchicine, bottom panels). Note the disappearance of the bipolar spindle after treatment with colchicine. Time: min:sec. Scale Bar=5 µm
Supplementary figure 4

A

B
**Figure S4. GFP::LacI::Mre11 co-localizes with mRFP::Rad50 on laser-induced DSBs during mitosis.** (A) Time-lapse images of neuroblasts expressing GFP::LacI::Mre11 (Top row, red in merge) and mRFP::Rad50 (middle row, cyan in merge) after microirradiation (IR). The yellow arrowhead indicates the site of IR. The red arrows point to the GFP::LacI::Mre11 signal on DSB and the cyan arrows indicate mRFP::Rad50 signal at DSB. Note that GFP::LacI::Mre11 and mRFP::Rad50 signals were both detected on DSB within 20 seconds following laser ablation. AO: anaphase onset. Time: min:sec. Scale Bar=5 µm. In neuroblasts expressing GFP::LacI::Mre11 and mRFP::Rad50, both proteins co-localize in a large cytoplasmic aggregate present in all cells (white arrow in merge). (B) Time-lapse images of irradiated (IR) neuroblasts expressing GFP::LacI::Mre11 (Top row, red in merge) and mCherry::Polo (middle row, cyan in merge) and carrying LacO arrays on the X chromosome. The yellow arrowhead indicates the site of irradiation (IR). The red and white arrows indicate the GFP::LacI::Mre11 signal on the DNA lesions and LacO arrays respectively. The cyan arrows indicate mCherry::Polo signal at site of IR. Note that GFP::LacI::Mre11 is detected at the site of IR prior to mCherry::Polo (20 and 40sec respectively). AO: anaphase onset. Time=min:sec. Scale Bar=5 µm.
**Supplementary figure 5**

**Figure S5. Okra does not localize on laser-induced DSBs during mitosis.** Time lapse images of neuroblasts expressing H2A::mRFP and Okra::GFP after laser ablation. The yellow arrowheads indicate the site of IR. Cell #1 is in mitosis and cell #2 is in interphase. Cells are monitored within a few seconds after laser ablation (time 0:00). Okra::GFP signal appears after 3 minutes following DNA damage in the interphase nuclei (white arrow). In contrast, no Okra::GFP signal is detected on DNA breaks in mitotic cells. The white dashed lines highlight the contour of the cells. Time: min:sec. Scale Bar=5µm.
Movie 1. GFP::Polo kinetics at laser-induced DSBs created during prometaphase. Time-lapse video of wild type third instar larvae neuroblast expressing H2Az::mRFP (red) and GFP::Polo (Cyan). The first image represents the cell few second before irradiation. The yellow arrow points to the site of irradiation (IR). The white arrow indicates the appearance of GFP::Polo at the site of damage. Time 0:00 corresponds to the time of recording few seconds after irradiation. Time=min:sec. Images are maximum projections. The movie corresponds to Fig. 1.

Movie 2: GFP::Polo kinetics at laser-induced DSBs created during metaphase. Time-lapse video of wild type neuroblast expressing H2Az::mRFP (red) and GFP::Polo (Cyan). The first image represents the cell few second before irradiation. The yellow arrow points to the site of irradiation (IR). The white arrow indicates the appearance of GFP::Polo at the site of damage. Time 0:00 corresponds to the time of recording few seconds after irradiation. Time=min:sec. Images are maximum projections. The movie corresponds to Fig. 1.
Movie 3: GFP::Polo kinetics at laser-induced DSBs created during anaphase. Time-lapse video of wild type neuroblast expressing H2Az::mRFP (red) and GFP::Polo (Cyan). The images before time 0:00 correspond to the cell before irradiation. The yellow arrow points to the site of irradiation (IR). The white arrow indicates the appearance of GFP::Polo at the site of damage. Time 0:00 corresponds to the time of recording few seconds after irradiation. Time=min:sec. Images are maximum projections. The movie corresponds to Fig. 1.

Movie 4: Polo kinase activity is required for its robust recruitment to mitotic DSBs. Time-lapse video of wild type neuroblast expressing H2Az::mRFP (red) and GFP::Polo (Cyan) previously treated with Colchicine (left panel) or BI2536 (right panel) for 30 minutes. The first image corresponds to pre-irradiation. The yellow arrows point to the site of irradiation (IR). The white arrows indicate the appearance of GFP::Polo at the site of damage for the indicated treatment. Time 0:00 corresponds to the time of recording few seconds after irradiation. Time=min:sec. Images are maximum projections. The movie corresponds to Fig. 2A.
**Movie 5: Kinetics of GFP::BubR1 and mCherry::Polo to DSBs during mitosis.** Time-lapse video of wild type neuroblast expressing GFP::BubR1 (Cyan) and mCherry::Polo. The first image represents the cell pre-irradiation. The yellow arrows point to the site of irradiation (IR). The red and cyan arrows indicate the appearance of mCherry::Polo and GFP::BubR1 respectively at the site of damage. Time 0:00 corresponds to the time of recording few seconds after irradiation. Time=min:sec. Images are maximum projections. The movie corresponds to Fig. 4A.

**Movie 6: GFP::BubR1 Kinetics at mitotic DSBs is altered in polo mutant.** Time-lapse video of wild type neuroblast arrested in prometaphase after colchicine treatment (left panel) and polo10 mutant neuroblasts (right panel) expressing H2Az::mRFP (red) and GFP::BubR1 (cyan). The first image represents the cells pre-irradiation. The yellow arrows point to the site of irradiation (IR). The white arrows indicate the appearance of GFP::BubR1 at the site of damage. Time 0:00 corresponds to the time of recording few seconds after irradiation. Time=min:sec. Images are maximum projections. The movie corresponds to Fig. 5A (top panels).
Movie 7: GFP::Bub3 Kinetics at mitotic DSBs is altered in polo mutant. Time-lapse video of wild type neuroblast arrested in prometaphase after colchicine treatment (left panel) and polo
mutant neuroblasts (right panel) expressing H2Az::mRFP (red) and GFP::Bub3 (cyan). The first image represents the cells pre-irradiation. The yellow arrows point to the site of irradiation (IR). The white arrows indicate the appearance of GFP::Bub3 at the site of damage. Time 0:00 corresponds to the time of recording few seconds after irradiation. Time=min:sec. Images are maximum projections. The movie corresponds to Fig. 5A (bottom panels).
Movie 8: GFP::Mre11 Kinetics at mitotic DSBs. Time-lapse video of wild type neuroblast expressing H2Az::mRFP (red) and GFP::Mre11 (cyan). The first image represents the cell pre-irradiation. The yellow arrow points to the site of irradiation (IR). The white arrow indicates the appearance of GFP::Mre11 at the site of damage. Time 0:00 corresponds to the time of recording few seconds after irradiation. Time=min:sec. Images are maximum projections. The movie corresponds to Fig. 6A (top panels).
Movie 9: mRFP::Rad50 Kinetics at mitotic DSBs. Time-lapse video of wild type neuroblast expressing H2Az::GFP (red) and mRFP::Mre11 (cyan). The first image represents the cell pre-irradiation. The yellow arrow points to the site of irradiation (IR). The white arrow indicates the appearance of mRFP::Rad50 at the site of damage. Time 0:00 corresponds to the time of recording few seconds after irradiation. Time=min:sec. Images are maximum projections. The movie corresponds to Fig. 6A (bottom panels).
**Movie 10: GFP::Polo Kinetics at mitotic DSBs is altered in rad50 mutant.** Time-lapse video of wild type (left panel) and rad50EP mutant (right panel) neuroblasts expressing H2Az::mRFP (red) and GFP::Polo (cyan). The first image represents the cells pre-irradiation. The yellow arrows point to the site of irradiation (IR). The white arrows indicate the appearance of GFP::Polo at the site of damage. Time 0:00 corresponds to the time of recording few seconds after irradiation. Time=min:sec. Images are maximum projections. The movie corresponds to Fig. 7B.