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Polo-mediated phosphorylation of Maelstrom regulates oocyte determination during oogenesis in *Drosophila*

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SUMMARY

In *Drosophila*, Maelstrom is a conserved component of the perinuclear nuage, a germline-unique structure that appears to serve as a site for Piwi-interacting RNA (piRNA) production to repress deleterious transposons. Maelstrom also functions in the nucleus as a transcriptional regulator to repress the expression of microRNA-7, a process that is essential for the proper differentiation of germline stem cells. In this paper, we report another function of Maelstrom in regulating oocyte determination independently of its transposon silencing and germline stem cell differentiation activities. In *Drosophila*, the conserved serine 138 residue in Maelstrom is required for its phosphorylation, an event that promotes oocyte determination. Phosphorylation of Maelstrom is required for the repression of the pachytene checkpoint protein Sir2, but not for transposon silencing or for germline stem cell differentiation. We identify Polo as a kinase that mediates the phosphorylation of Maelstrom. Our results suggest that the Polo-mediated phosphorylation of Maelstrom may be a mechanism that controls oocyte determination by inactivating the pachytene checkpoint via the repression of Sir2 in *Drosophila* ovaries.

KEY WORDS: Maelstrom, *Drosophila*, Nuage, Polo, Sir2, Pachytene checkpoint, Germline, Phosphorylation

INTRODUCTION

Maelstrom (Mael) is an evolutionarily conserved protein that contains a high-mobility group (HMG) box and a putative 3′-5′ exoribonuclease domain (Findley et al., 2003; Zhang et al., 2008). In both *Drosophila* and mice, Mael is required for the production of Piwi-interacting RNAs (piRNAs) (Lim and Kai, 2007; Soper et al., 2008; Aravin et al., 2009) that belong to a gonadal-specific class of small RNAs (23-29 nucleotides) required for the silencing of transposons and other developmental processes (Khurana and Theurkauf, 2010; Senti and Brennecke, 2010; Siomi et al., 2011; Pek et al., 2012a). Similar to many piRNA pathway proteins, Mael localises to the nuage, a germline-unique structure that is proposed to be the site of piRNA processing during the secondary amplification cycle: the Ping-Pong cycle (Findley et al., 2003; Voronina et al., 2011; Pek et al., 2012a; Costa et al., 2006; Siomi et al., 2011). However, the molecular function of Mael in the piRNA pathway remains unclear.

In *Drosophila*, Mael also localises to the nucleus, in which it promotes germline stem cell (GSC)/cystoblast (CB) differentiation by repressing the expression of miRNA-7 (miR-7) (Findley et al., 2003; Pek et al., 2009). A recent study has implicated another role for Mael in regulating the microtubule-organising centre (MTOC) by interacting with the components of the MTOC in *Drosophila* ovaries (Sato et al., 2011). Therefore, Mael appears to perform multiple functions during gametogenesis. An understanding of the mechanism(s) through which Mael is regulated will provide important insights into its specific functions.

Recent studies have shown that some piRNA pathway proteins, including those that localise to the nuage, are regulated by post-translational modifications (PTMs). These proteins comprise of the Piwi family proteins, including Piwi, Aubergine (Aub) and Argonaute3 (Ago3), and the nuage protein Vasa. For example, *Drosophila* Piwi, Aub, Ago3 and Vasa contain symmetrically dimethylated arginines that promote their binding to some Tudor domain proteins (Kirino et al., 2009; Kirino et al., 2010; Nishida et al., 2009; Chen et al., 2011; Pek et al., 2012b). Both Vasa and Piwi have also been shown to be phosphorylated in *Drosophila* ovaries (Ghabrial and Schüpbach, 1999; Gangaraju et al., 2011). Although the phosphorylation of Vasa regulates the DNA damage checkpoint, the kinase that mediates this phosphorylation event remains unknown. Furthermore, the biological significance of Piwi phosphorylation is currently unclear. A recent study has also shown that the mouse Vasa homologue (MVH) is regulated by acetylation (Nagamori et al., 2011). As Mael is a piRNA pathway component that localises to the nuage, it is possible that its activity is also regulated by any PTMs.

Two meiotic checkpoints function in *Drosophila*: the double-strand break-dependent DNA damage checkpoint and the double-strand break-independent pachytene checkpoint (Joyce and McKim, 2011; Lake and Hawley, 2012). The upregulation of transposons in piRNA pathway mutants has been implicated to trigger the DNA damage checkpoint that is mediated by Vasa phosphorylation (Ghabrial and Schupbach, 1999; Klattenhoff et al., 2007; Chen et al., 2007). However, oocyte determination defects in nuage/piRNA pathway mutants have been shown to be regulated by a DNA damage checkpoint-independent mechanism (Blumenstiel et al., 2008). The oocyte determination phenotype is characteristic of the activation of the pachytene checkpoint that is often triggered by a failure of chromosomal synapsis (Joyce and McKim, 2010). However, it is currently unclear whether piRNA pathway or nuage component(s) play a role in regulating oocyte determination through the pachytene checkpoint.

In this paper, we show that the Polo kinase promotes the phosphorylation of Mael in *Drosophila* ovaries, and that this phosphorylation is required for oocyte determination but not for transposon silencing or GSC/CB differentiation. We propose that

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the Polo-mediated phosphorylation of Mael is required for the repression of the pachytene checkpoint in part through the downregulation of the checkpoint protein Sir2.

MATERIALS AND METHODS

Drosophila strains

In this study, the following strains were used: *maelM391/Df(3L)79E-F* (Findley et al., 2003), *UASp-FLAG-mael* (Pek et al., 2009), *polo-GFP* (Moutinho-Santos et al., 1999), $polo^{1}$ (Tavares et al., 1996), $polo^{9}$ (Deák et al., 1997), $sir2^{17}$ (Aström et al., 2003), $C(2)M^{EP2115}$ (Manheim and McKim, 2003) and $pch2^{EY01788a}$ (Joyce and McKim, 2009). *y w* or heterozygous flies were used as the controls. The Mael-S138A and Mael-S138D mutations were generated using the QuickChange II XL site-directed mutagenesis kit (Stratagene) with pPFW-mael (Pek et al., 2009) as the template and the following sets of primers: mael_S138A_Fw (5′-GCGGCATGCGAATATGCGTTGAAGGAGGGAATC-3′) and mael_S138A_Rv (5′-GATTCCCTCCTTCAACGCATATTCGCATGCC-GC-3′); and mael_S138D_Fw (5′-GCGGCATGCGAATATGACTTGAAG-GAGGGAATC-3′) and mael_S138D_Rv (5′-GATTCCCTCCTTCAAG-TCATATTCGCATGCCGC-3′). Both the *UASp-FLAG-mael-S138A* and *UASp-FLAG-mael-S138D* transgenic flies were generated as previously described (Rubin and Spradling, 1982).

Immunostaining

Immunostaining was performed as described previously (Pek and Kai, 2011b; Pek and Kai, 2011a). The following primary antibodies were used: guinea pig anti-Vasa (1:1000) (Patil and Kai, 2010), mouse anti-FLAG (1:1000, Sigma), mouse anti-Hts monoclonal 1B1 (1:5, DSHB), mouse anti-C(3)G (1:500, a kind gift from Scott Hawley, Stowers Institute for Medical Research, Kansas Ciry, MO, USA), mouse anti-Sir2 monoclonal p4A10 (1:20, DSHB), rabbit anti-alpha spectrin (1:1000) (Byers et al., 1987), rabbit anti-CID (1:500, Abcam), rabbit anti-GFP (1:200, Abcam), mouse anti-γH2Av (1:100, Millipore) and guinea pig anti-Mael (1:500) (Pek et al., 2009). Images were obtained using the Carl Zeiss LSM5 Exciter Upright microscope and processed with Photoshop.

Western blotting

Western blot analyses were performed as described previously (Pek and Kai, 2011b; Pek and Kai, 2011a). The primary antibodies used were as follows: mouse anti-FLAG (1:1000, Sigma), mouse anti-alpha tubulin (1:1000, Upstate), rabbit anti-GFP (1:1000, Clontech), rabbit anti-Sir2 (1:200, Clontech) and rabbit anti-Casein kinase 2a (CK2a) (1:1000, Abcam). Phosphorylated Mael was detected using PhosTag SDS-PAGE (Wako) (Kinoshita et al., 2006) that allows phosphorylated forms of proteins to be identified because the lower-mobility forms migrate slower than the non-phosphorylated proteins.

Phosphatase treatment

The ovaries from ten flies were dissected in cold Grace's medium and homogenised in ice-cold PBS. The lysate was then incubated in reaction buffer and calf intestinal alkaline phosphatase (CIAP) (Promega) for 2 hours at 37°C with occasional mixing. The reaction was stopped by the addition of 2× sample buffer and the mixture was boiled for 5 minutes.

Prediction of putative phosphorylation sites

The NetPhos prediction program (http://www.cbs.dtu.dk/services/NetPhos/) (Blom et al., 1999) was used to predict the putative phosphorylation sites in Mael. Only those residues with scores of more than or equal to 0.95 were selected.

RT-PCR

Semi-quantitative RT-PCR was performed as described previously (Lim and Kai, 2007) using previously reported primers (Lim and Kai, 2007; Pek et al., 2009).

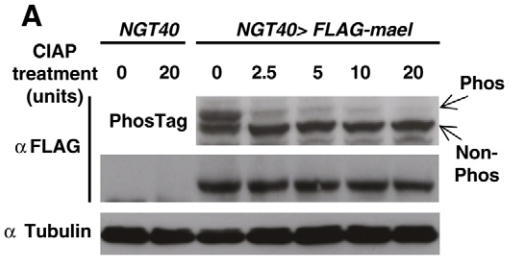
Co-immunoprecipitation

Co-immunoprecipitation was performed as described previously (Pek and Kai, 2011b; Pek and Kai, 2011a). The antibody used for immunoprecipitation was mouse anti-FLAG (Sigma). The input corresponded to 1.0% of the lysates.

RESULTS

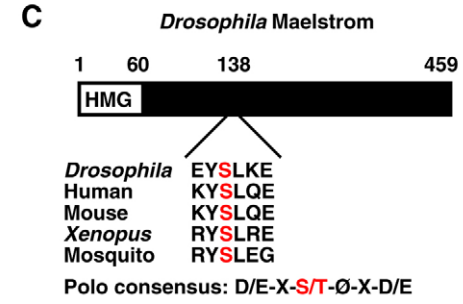
Maelstrom is phosphorylated in *Drosophila* ovaries

To understand the mechanism through which Mael is regulated, we examined whether Mael is phosphorylated in vivo by expressing *NGT40*-driven FLAG-Mael in ovarian germline cells and examined its phosphorylation using PhosTag SDS-PAGE (Kinoshita et al., 2006). FLAG-Mael migrated as a doublet, suggesting that Mael is phosphorylated (Fig. 1A). To confirm this observation, we treated the ovarian lysate expressing FLAG-Mael with calf intestinal alkaline phosphatase (CIAP) and subjected it to PhosTag SDS-PAGE. The lower-mobility form of FLAG-Mael (phosphorylated) was converted to the higher-mobility form (non-phosphorylated) in a CIAP dose-dependent manner (Fig. 1A), indicating that Mael is phosphorylated in the ovaries.



B

Position	Sequence	Score	Conserved
67	AKRESSNGH	0.991	No
68	KRESSNGHG	0.902	No
84	EATESLMDM	0.990	No
138	ACEYSLKEG	0.950	Yes
198	VDYLSKCQG	0.942	No
346	SSYASSLDH	0.996	No
352	LDHESSFDS	0.971	No
369	VKKESQPEA	0.993	No
382	SSRLSVASS	0.995	No
387	VASSSYKPI	0.994	No
415	MRNSSKHHG	0.961	No
444	IRKVSDNDF	0.989	No
27	GRRMTLAQA	0.963	No



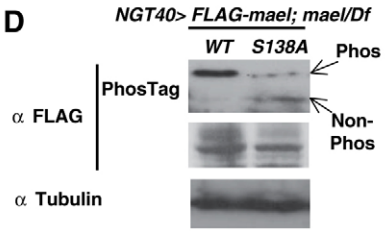


Fig. 1. *Drosophila* Mael is phosphorylated in the ovaries. (**A**) Ovary lysates were treated with CIAP and subjected to PhosTag or normal SDS-PAGE. A phosphorylated FLAG-Mael band of lower mobility was converted to a non-phosphorylated form of higher mobility by CIAP treatment. (**B**) The putative phosphorylated amino acids of Mael. The table shows the positions of the amino acids (in red) predicted by NetPhos to have a score higher than or equal to 0.95 and their evolutionary conservation. (**C**) S138 of Mael is conserved in human, mouse, *Xenopus* and mosquito. (**D**) Wild-type and Mael-S138A were expressed in the germline cells in the *mael* mutant background and subjected to PhosTag or normal SDS-PAGE. The phosphorylated FLAG-Mael band of lower mobility was greatly reduced in Mael-S138A-expressing ovaries.

We next searched for potential phosphorylation sites of Mael. Many putative sites were identified with NetPhos, a program that predicts putative phosphorylation sites (Blom et al., 1999). Two criteria were used to narrow the search: a NetPhos score more than or equal to 0.95; and evolutionary conservation of the residue, implicating its biological importance. Among the putative amino acids, serine 138 (S138) was the only one that fulfilled these two criteria (Fig. 1B,C). Further analysis indicated that the amino acid sequence that flanks S138 of *Drosophila* Mael (EYSLKE) matches the consensus sequence for Polo kinase (D/E-X-S/T-Ø-X-D/E) (Fig. 1C) (Nakajima et al., 2003). To examine whether S138 is required for Mael phosphorylation, we generated FLAG-Mael-S138A in which S138 is mutated to a non-phosphorylatable alanine. The expression of wild-type FLAG-Mael and FLAG-Mael-S138A in *mael* mutant ovaries showed that FLAG-Mael-S138A resulted in a marked reduction in the lower-mobility (phosphorylated) form of FLAG-Mael (Fig. 1D). This result indicates that S138 is required for the robust phosphorylation of Mael in vivo. However, some residual phosphorylated forms of Mael remained (Fig. 1D), suggesting that Mael may also be phosphorylated at other residue(s) in addition to S138. We cannot exclude the possibility that this trace amount of phosphorylated Mael may be due to an artefact of transgenic Mael expression. Taken together, our data suggest that S138 is a potential phosphorylation site of Mael.

We next examined whether the phosphorylation of Mael is required for its perinuclear and nuclear localisation by analysing the localisation of tagged Mael variants, FLAG-Mael-S138A (a non-phosphorylatable form) and FLAG-Mael-S138D (a phosphomimetic form). Driven by *NGT40*, these tagged proteins were expressed in germline cells at similar levels to wild-type FLAG-Mael (supplementary material Fig. S1). Tagged Mael variants were localised to the perinuclear nuage (co-localising with Vasa) and nucleus (Fig. 2), indicating that phosphorylation does not regulate the localisation.

Phosphorylation of Maelstrom is required for oocyte determination but not for transposon silencing or GSC differentiation

Because Mael functions via the piRNA pathway to silence transposons in *Drosophila* ovaries (Lim and Kai, 2007; Klenov et al., 2011), we next examined whether the phosphorylation of Mael is required for its transposon silencing activity. Consistent with previous findings, the *TART*, *I element* and *jockey* transposons were robustly de-repressed in *mael* mutant ovaries (Fig. 3A) (Lim and Kai, 2007; Klenov et al., 2011). However, the *NGT40*-driven expression of either FLAG-Mael-S138A or FLAG-Mael-S138D in the germline cells efficiently repressed the transposon expression to levels that were comparable with the heterozygous controls (Fig. 3A). The de-repression of transposons in piRNA pathway mutants is thought to lead to an increase in DNA double-strand breaks (Klattenhoff et al., 2007; Patil and Kai, 2010). Consistent with the repression of transposons by both of the Mael transgenes, the accumulation of double-strand breaks in *mael* mutant oocyte nuclei was also rescued, as visualised by the double-strand break marker phosphorylated H2Av (supplementary material Fig. S2). Therefore, our results suggest that the phosphorylation of Mael does not appear to be necessary for its transposon silencing activity.

It has been shown that the nuclear activity of Mael is required for repressing primary miR-7 (pri-miR-7) expression to ensure proper germline stem cell differentiation (Pek et al., 2009). We next examined whether the phosphorylation of Mael is involved in repressing pri-miR-7 expression by examining the pri-miR-7 levels in *mael* mutant ovaries expressing the Mael variant transgenes. Although pri-miR-7 was upregulated in the *mael* mutant ovaries, it became repressed to a level comparable with the heterozygous controls when either FLAG-Mael-S138A or FLAG-Mael-S138D was expressed in the germline cells (Fig. 3B). Consistent with the rescue of the upregulated pri-miR-7 phenotype, the GSC differentiation defect in the *mael* mutant ovaries was also rescued by the germline expression of either FLAG-Mael-S138A or FLAG-Mael-S138D (Fig. 3C,D). In the heterozygous control ovaries, we

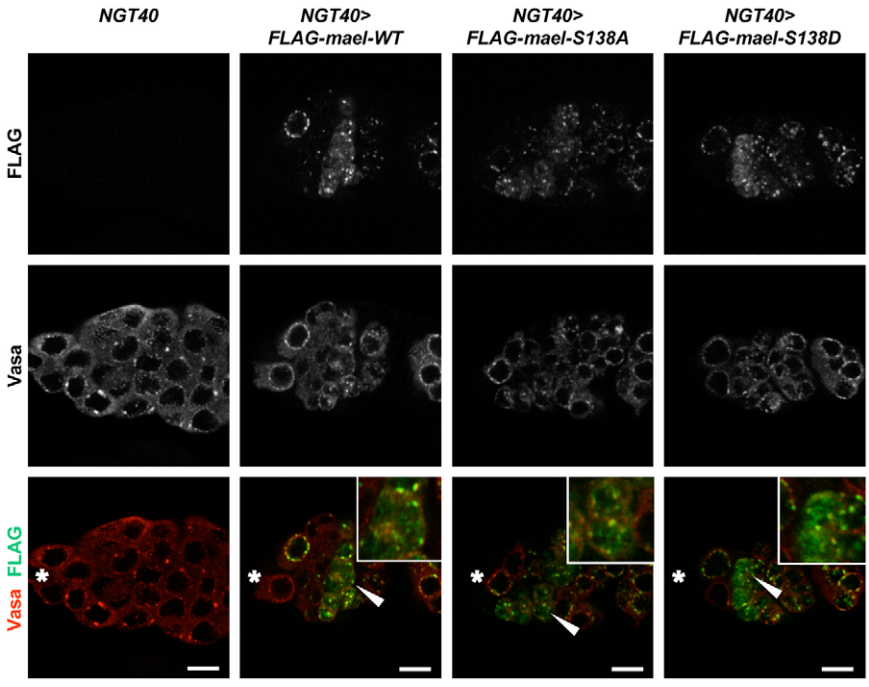


Fig. 2. Phosphorylation of Mael is not required for its nuclear and perinuclear localization. Germaria harbouring transgenes of the indicated genotypes were stained for FLAG-Mael (green) and Vasa (red). Insets show the magnified nuclei (arrowheads). Asterisks indicate anterior tips of germaria. Scale bars: 10 μm.

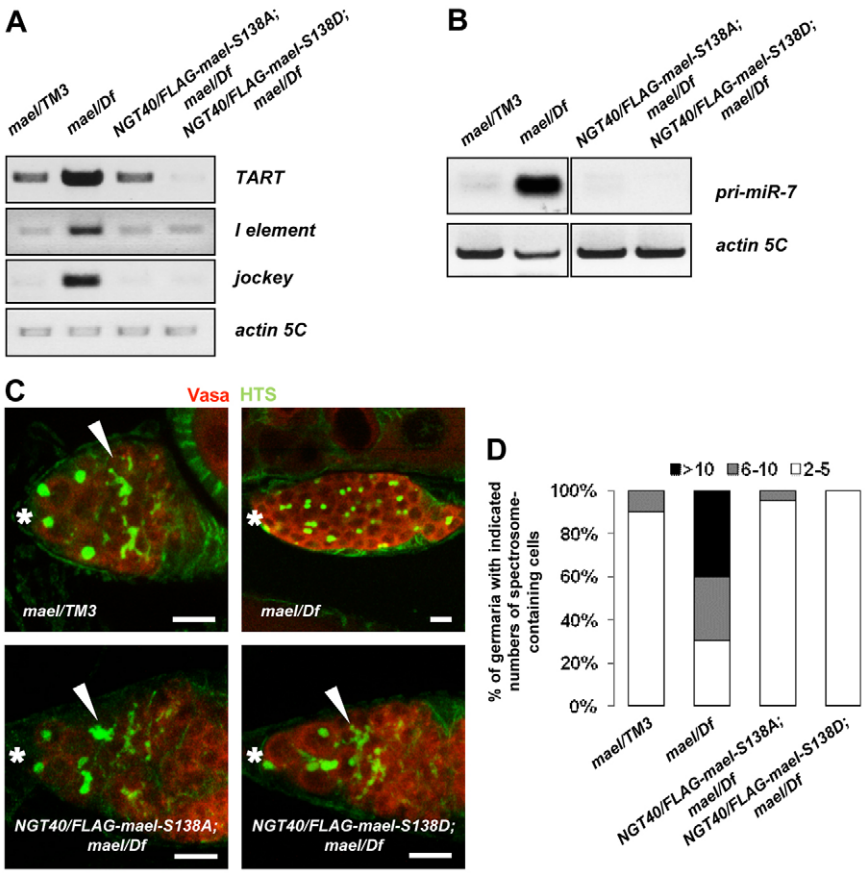


Fig. 3. Phosphorylation of Mael is not transposon silencing or GSC/CB differentiation. (**A**) RT-PCR showing the expression of transposons in the ovaries of the indicated genotypes. (**B**) RT-PCR showing the expression of primary *miR-7* in the ovaries of the indicated genotypes. (**C**) Germaria of the indicated genotypes stained for Hts (green) and Vasa (red) to visualise fusomes and germline cells. The arrowheads indicate differentiating cysts containing branched fusomes. Asterisks indicate anterior tips of germaria. Scale bars: 10 μm. (**D**) Quantification of germaria with the indicated number of spectrosome-containing cells (n>20).

usually observed between approximately two and five spectrosome-containing GSCs/CBs (Fig. 3D) and the robust differentiation of cysts, as shown by the presence of branched fusomes (Fig. 3C, arrowheads) (de Cuevas and Spradling, 1998). However, in the *mael* mutant ovaries, the spectrosome-containing GSCs/CBs accumulated in ~70% of the germaria with more than six GSCs/CBs (Fig. 2D); this observation was concomitant with fewer differentiating cysts (Fig. 3C), indicating a GSC/CB differentiation defect (Pek et al., 2009). These defects were rescued by both FLAG-Mael-S138A and FLAG-Mael-S138D: the number of GSCs/CBs returned to normal (Fig. 3D) and a robust differentiation of cysts was observed (Fig. 3C, arrowheads). Therefore, we concluded that the phosphorylation of Mael is not important for its nuclear function in promoting GSC/CB differentiation.

We further addressed whether the phosphorylation of Mael is required for proper oocyte determination during oogenesis. Previous reports have shown that some nuage/piRNA pathway mutants, including *mael*, *aub*, *spindle-E* (*spn-E*) and *tejas*, display oocyte determination defects (Findley et al., 2003; Blumenstiel et al., 2008; Huynh and St Johnston, 2000; Patil and Kai, 2010; Barbosa et al., 2007). Using C(3)G staining, which labels the meiotic chromosomes of both pro-oocytes and oocytes, we examined the requirement of Mael phosphorylation in oocyte determination. In the heterozygous controls, the oocytes were already determined during late pachytene at region 3/stage 1 in ~70% of the germaria (Fig. 4) (Joyce and McKim, 2010). Consistent with previous reports, the 'two-oocyte phenotype' was evident in ~80% of the germaria in the *mael* mutants (Fig. 4), indicating a delay in oocyte commitment (Findley et al., 2003; Sato et al., 2011). Expression of the wild-type FLAG-Mael transgene suppressed the phenotype to ~20% (Fig. 4). The expression of FLAG-Mael-S138A in the germline cells did not suppress the 'two-oocyte phenotype' that remained high at ~70% (Fig. 4). By contrast, the germline expression of FLAG-Mael-S138D partially suppressed the phenotype to ~50% of the germaria (Fig. 4), suggesting that the phosphorylation of Mael is required for proper oocyte determination during the pachytene stage. Furthermore, expression of either the wild-type or FLAG-Mael-S138D, but not the FLAG-Mael-S138A transgene, significantly restored the egg-laying rate (supplementary material Fig. S3). Taken together, our results suggest that Mael may play a role in regulating oocyte determination independently of its activity in transposon silencing and GSC/CB differentiation.

Polo is required for Mael phosphorylation

Because the amino acid sequence flanking S138 matches the consensus target sequence of the Polo kinase, we next investigated whether Polo functions in the phosphorylation of Mael. Previous studies have shown that Polo regulates various aspects of mitosis in *Drosophila* by dynamically localising to the centrosomes, centromeres and microtubules (Llamazares et al., 1991; Moutinho-Santos et al., 1999; Logarinho and Sunkel, 1998), whereas Polo localises as discrete perinuclear foci in the germline cells of the germarium during interphase (Mirouse et al., 2006). We also observed the perinuclear localisation of Polo-GFP, in which some of the signals overlapped and/or were juxtaposed with Mael foci (Fig. 5A). To examine whether the Polo kinase interacts with Mael, we performed co-immunoprecipitation assays using ovarian lysates

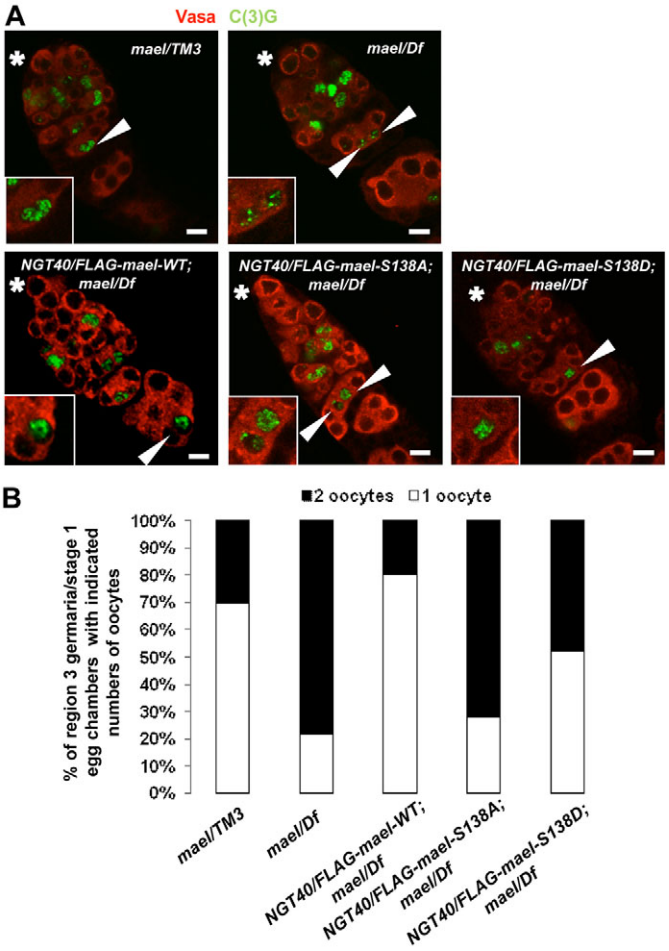


Fig. 4. Phosphorylation of Mael is required for proper oocyte determination. (**A**) Germaria of the indicated genotypes stained for the synaptonemal marker C(3)G (green) to visualise pro-oocyte and/or oocyte nuclei and Vasa (red) to visualise germline cells. The arrowheads indicate pro-oocytes/oocytes at region 3 of the germaria. Asterisks indicate anterior tips of germaria. Scale bars: 10 μm. (**B**) Quantification of germaria with the indicated number of oocytes (*n*>20).

expressing FLAG-Mael in germline cells. The *NGT40*-driven expression of FLAG-Mael occurred predominantly at region 2 of the germarium (Fig. 2), making it a suitable system with which to detect Polo-Mael associations during pachytene. Polo-GFP, but not the other kinase, CK2a, was co-immunoprecipitated with FLAG-Mael (Fig. 5B), suggesting that Polo and Mael may form a complex in the germline cells. In addition, phosphorylated FLAG-Mael was greatly reduced in $polo^{1}/polo^{9}$ mutant ovaries compared with that in the wild-type controls (Fig. 5C). Our results suggest that Polo appears to interact with Mael to mediate the phosphorylation of Mael.

We next examined whether the phosphorylation of Mael mediated by Polo regulates oocyte determination. Because *polo* homozygous null allele mutants are embryonic lethal, we used the transheterozygous $polo^{1}/polo^{9}$ alleles that were used in previous studies (Mirouse et al., 2006; Clark et al., 2005). The percentage of cysts in the region 3/stage 1 egg chambers containing two oocytes increased from ~25% in the heterozygous controls to ~90% in the *polo* mutants (Fig. 5D,E), suggesting that *polo* is required for proper oocyte determination. Interestingly, the germline expression of FLAG-Mael-S138D but not FLAG-Mael-S138A reduced the frequency of two oocytes in the *polo* mutants (~90% for FLAG-Mael-S138A versus ~50% for FLAG-Mael-S138D; Fig. 5D,E), further supporting the hypothesis that Polo promotes oocyte commitment in *Drosophila* ovaries by mediating the phosphorylation of Mael. Consistent with our observation that the phosphorylation of Mael is not required for transposon repression or GSC/CB differentiation, the *polo* mutants did not exhibit transposon de-repression or GSC/CB differentiation phenotypes (supplementary material Fig. S4).

Phosphorylation of Mael represses the pachytene checkpoint

The ‘two-oocyte phenotype’ is characteristic of the activation of the pachytene checkpoint that is often triggered by a failure of chromosomal synapsis (Joyce and McKim, 2010). We examined whether the phosphorylation of Mael regulates oocyte determination by repressing the pachytene checkpoint. Sir2 is an evolutionarily conserved histone deacetylase that regulates the meiotic checkpoint (MacQueen and Hochwagen, 2011). In *Drosophila*, *sir2* is required for the activation of the pachytene checkpoint (Joyce and McKim, 2010; Joyce and McKim, 2009). To address whether Sir2 is regulated by Mael during meiosis, we immunostained the control and *mael* mutant germaria for Sir2. In the heterozygous controls, Sir2 was highly expressed in regions 1 and 3 but dramatically downregulated in regions 2a and 2b (Fig. 6A,B). The downregulation of Sir2 during the early and mid-pachytene stages in regions 2a and 2b is consistent with the suppression of the pachytene checkpoint in wild-type flies. In the *mael* mutant ovaries expressing FLAG-Mael-S138A with a prevalent ‘two-oocyte phenotype’ (Fig. 4), the expression of Sir2 remained high in regions 2a and 2b, an observation that was also present in the meiotic mutant *C(2)M* (Fig. 6B), suggesting an activation of the pachytene checkpoint. However, the high expression of Sir2 in regions 2a and 2b was not observed in the *mael* mutant ovaries expressing either FLAG-Mael-WT or FLAG-Mael-S138D (Fig. 6A,B), in which oocyte determination occurred normally (Fig. 4). Through western blotting of lysates from hand-dissected germaria, we confirmed that high levels of Sir2 protein were present in the germaria of the *mael* mutants expressing FLAG-Mael-S138A compared with the heterozygous controls and *mael* mutants expressing FLAG-Mael-S138D (Fig. 6C). Consistent with the role of Polo in mediating Mael phosphorylation, we also observed that Sir2 expression remained high in regions 2a and 2b in the *polo* mutant ovaries (Fig. 6A,B). However, high levels of Sir2 in *polo* mutant ovaries were not rescued by the expression of FLAG-Mael-S138D transgene (Fig. 6B). This observation possibly explains the partial rescue of the ‘two-oocyte’ phenotype in *polo* mutants by FLAG-Mael-S138D transgene (Fig. 5E) and further suggests that Polo may also regulate other target(s). Taken together, our data suggest that Polo mediates Mael phosphorylation that is required to repress the expression of Sir2 in region 2 during pachytene checkpoint inactivation.

To verify that the oocyte determination defect is caused by the high expression of Sir2 in *polo* mutants, we examined whether reducing the expression of Sir2 could rescue the phenotype. Removing one copy of *sir2* reduced the frequency of two oocytes in the *polo* mutants (~80% in the *polo* mutants versus ~25% in the *sir2/+, polo* mutants; Fig. 6D). Similarly, the depletion of another pachytene checkpoint component, *pch2*, also reduced the frequency of two oocytes in the *polo* mutants (25%, Fig. 6D). Taken together, our results suggest that the Polo-mediated phosphorylation of Mael

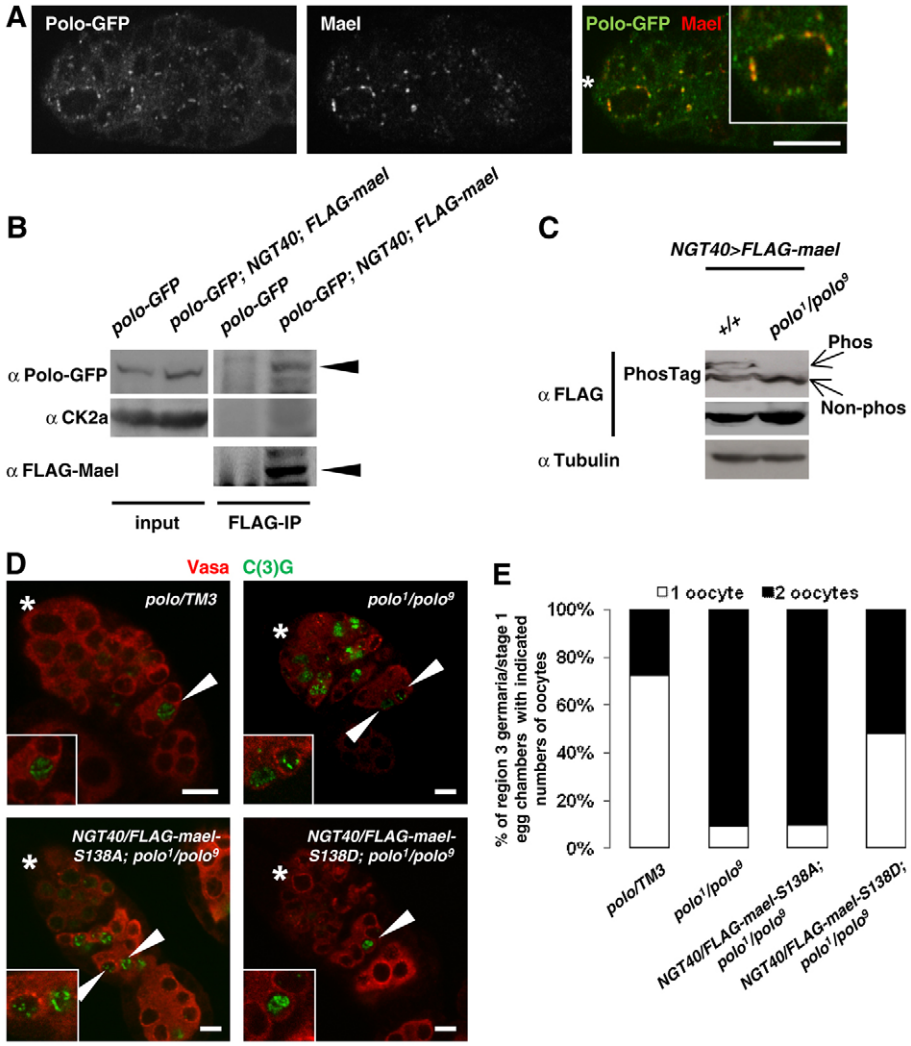


Fig. 5. Polo promotes proper oocyte determination by mediating Mael phosphorylation. (**A**) Polo localises to perinuclear foci that overlap and/or juxtapose with Mael. *polo-GFP* germarium stained for GFP to visualise Polo-GFP (green) and Mael (red). Inset shows a magnified GSC. Asterisk indicates anterior tips of germaria. (**B**) Western blots showing the co-immunoprecipitation of Polo-GFP and FLAG-Mael from ovarian lysates. Polo-GFP co-immunoprecipitated with FLAG-Mael but not in the control, which did not express FLAG-Mael. CK2a did not co-immunoprecipitate with FLAG-Mael, indicating its specificity. The arrowheads indicate co-immunoprecipitated proteins. (**C**) Western blots showing the reduction of the phosphorylated form of FLAG-Mael in *polo* mutant ovaries. (**D**) Germaria of the indicated genotypes stained for C(3)G (green) and Vasa (red). The arrowheads indicate pro-oocytes/oocytes at region 3 of the germaria. Asterisks indicate anterior tips of germaria. (**E**) Quantification of germaria with the indicated number of oocytes (n>20). Scale bars: 10 μm.

is required to repress the activation of the pachytene checkpoint in part by repressing the expression of Sir2.

We next examined whether the phosphorylation of Mael by Polo represses the pachytene checkpoint indirectly by regulating centromeric clustering during meiosis. During the pachytene stage, synapsis is initiated by the clustering of the centromeres to 1-3 bodies (Takeo et al., 2011). We examined the clustering of the centromeres in region 3/stage 1 of germaria through the immunostaining of CID (centromere identifier), a marker for centromeres (Blower and Karpen, 2001). In *C(2)M* mutant ovaries, where synapsis is defective, CID appeared as multiple foci (~70% with more than three foci) (Fig. 7A,B). However, in *mael* mutants expressing either FLAG-Mael-S138A or FLAG-Mael-S138D or in the *polo* mutants, we did not observe any significant defects in the clustering of the centromeres (Fig. 7A,B) (Tanneti et al., 2011; Takeo et al., 2011). In addition, whereas C(3)G staining appeared patchy in C(2)M mutants, C(3)G staining appeared chromosomal in the *mael* mutants expressing either FLAG-Mael-S138A or FLAG-Mael-S138D and in the *polo* mutants, similar to those in the controls (Fig. 7A,B), further suggesting that synapsis was normal in these mutants. As $polo^{1}/polo^{9}$ are hypomorphic mutants, we cannot completely exclude the possibility that residual *polo* activity remains. Therefore, we conclude that the phosphorylation of Mael mediated by Polo regulates the pachytene checkpoint independently of meiotic centromeric clustering.

DISCUSSION

Our study suggests that the phosphorylation of Mael is specifically required for its activity in promoting oocyte determination, possibly by repressing the pachytene checkpoint but not in transposon silencing or in GSC/CB differentiation (Fig. 7C). The molecular mechanisms through which Mael regulates oocyte determination via the pachytene checkpoint require further investigation. Although phosphorylation of Mael is required for proper oocyte determination (Fig. 4), the two-oocyte phenotype in *mael* mutants was not suppressed by reducing one copy of *sir2* or *pch2* (data not shown). This is consistent with the roles of Mael regulating other processes such as histone modification and regulation of the microtubule-organising centre (MTOC) that can also affect oocyte determination (Pek et al., 2009; Sato et al., 2011). One possibility is that Mael may regulate the production of a specific class of pachytene piRNAs that control the pachytene checkpoint. Unlike in mice, however, there is currently no evidence for the presence of pachytene piRNAs in *Drosophila* (Siomi et al., 2011). Furthermore, we did not observe any piRNA-related defects, such as the upregulation of transposons

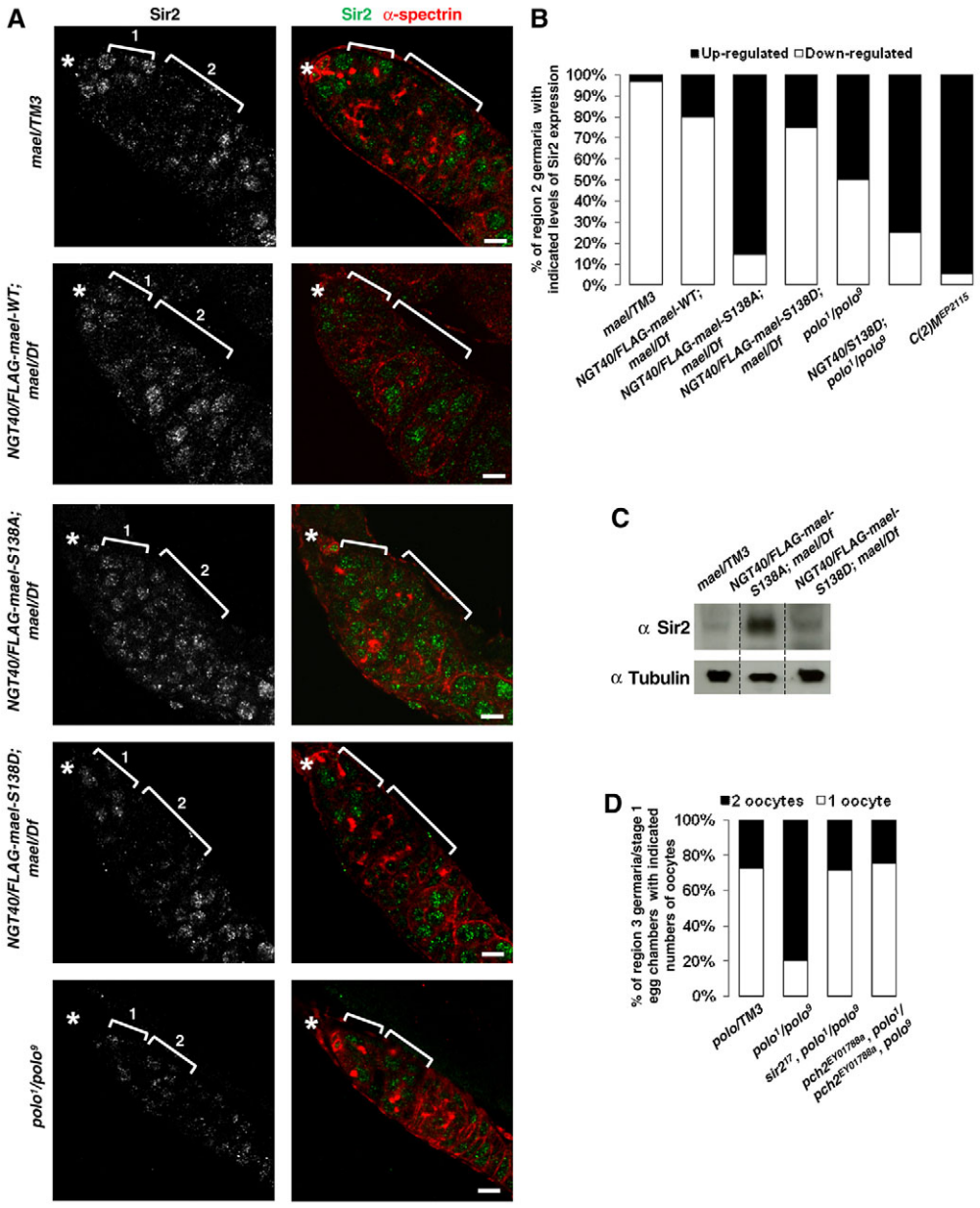


Fig. 6. The Polo-mediated phosphorylation of Mael represses the pachytene checkpoint. (**A**) Germaria of the indicated genotypes stained for Sir2 (green) and α-Spectrin (red) to visualise differentiating germline cells. The brackets depict regions 1 or 2 of the germaria. Asterisks indicate anterior tips of germaria. Scale bars: 10 μm. (**B**) Quantification of germaria exhibiting up- or downregulated Sir2 expression (n>20). (**C**) Western blots showing the upregulation of Sir2 in the germaria of *NGT40/FLAG-mael-S138A; mael/Df* flies. (**D**) *sir2* and *pch2* are required for the 'two-oocyte phenotype' in *polo* mutants. Quantification of germaria with the indicated number of oocytes (n>20).

or DNA double-strand breaks, in the *mael* mutants expressing the FLAG-S138A transgene (Fig. 3A; supplementary material Fig. S2), although these mutants exhibited defects in oocyte determination (Fig. 4). Additionally, consistent with a previous study reporting that piRNA pathway genes, such as *aub* and *spn-E*, are not required for homologous chromosome pairing during meiosis (Blumenstiel et al., 2008), we did not observe any defects in centromere clustering during synapsis in the *polo* and *mael* mutants expressing non-phosphorylatable form of Mael (Fig. 7A,B). Furthermore, as assayed by C(3)G staining, chromosomal axes formation was also unaffected in the *mael* and *polo* mutants when compared with that in the *C(2)M* mutants (Fig. 7A) (Joyce and McKim, 2010; Tanneti et al., 2011). Therefore, our results suggest that Mael may play a more direct role in regulating oocyte determination that is independent of chromosomal dynamics.

One possible mode of action of Mael may be to regulate the translation of pachytene checkpoint proteins, including Sir2, which was upregulated in *mael* mutants in a phosphorylation-dependent manner. The phosphorylation of Mael may regulate the activity of translation repression machineries, such as Bruno and Cup, which also localise to the nuage (Snee and Macdonald, 2004; Nakamura et al., 2004); this, in turn, may control the expression of Sir2. Future analyses will delineate the mechanistic function of Mael in regulating the pachytene checkpoint. It would be interesting to study the molecular mechanism by which Polo regulates Mael phosphorylation in response to meiotic defects.

In summary, we have identified Polo as the kinase responsible for Mael phosphorylation in *Drosophila* ovaries. Polo is highly regulated during oogenesis, demonstrating high expression in the germarium and repression until stage 11 of oogenesis (Mirouse et al., 2006; Xiang et al., 2007). Polo also interacts with BicD and is involved in oocyte determination (Mirouse et al., 2006). Therefore, Polo may play an important role in the pachytene checkpoint by controlling multiple targets, including Mael and BicD. A previous study has implicated the phosphorylation of Vasa, another nuage

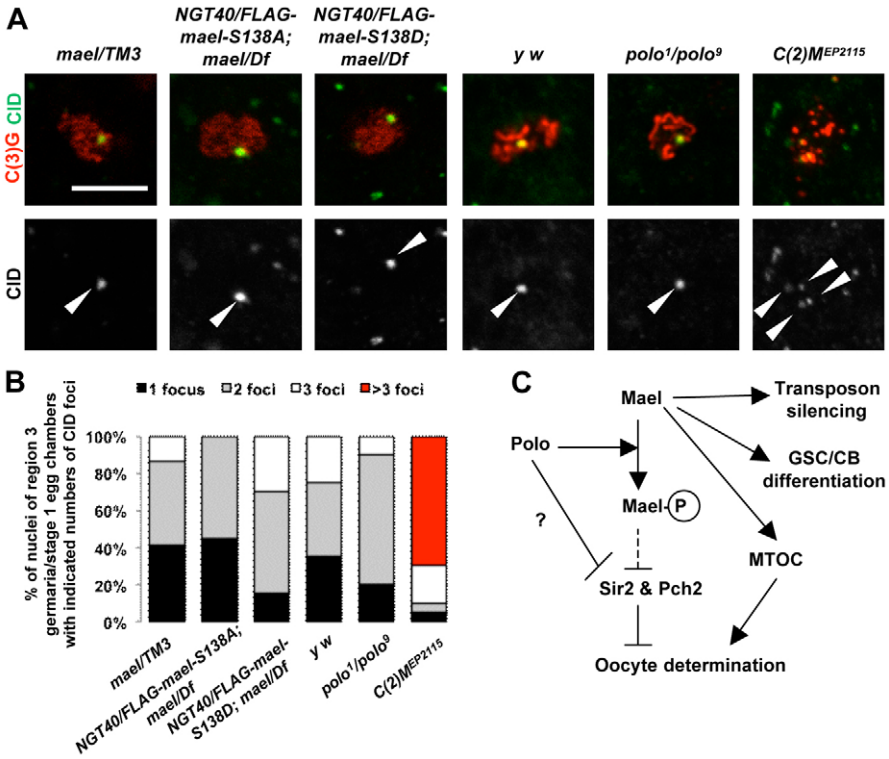


Fig. 7. The Polo-mediated phosphorylation of Mael does not regulate meiotic chromosomal dynamics. (**A**) Region 3 oocyte nuclei of the indicated genotypes stained for CID (green) to visualise the centromeres and C(3)G (red) to visualise the oocyte nuclei. The arrowheads indicate CID foci. Scale bar: 5 μm. (**B**) Quantification of the germaria with the indicated number of CID foci (n>20). *C(2)M* mutants were used as a positive control and demonstrated defective centromere clustering. (**C**) Proposed model of the regulation of oocyte determination by the Polo-mediated phosphorylation of Mael.

component, as a mechanism for regulating the DNA damage checkpoint in *Drosophila* ovaries (Ghabrial and Schüpbach, 1999). Based on our results, we propose another pathway of Polo-mediated Mael phosphorylation as a potential mechanism for the regulation of the pachytene checkpoint.

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