Title: M-Phase oscillations in PP1 activity are essential for accurate progression through mammalian oocyte meiosis

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Abbreviations:

PP1 – Protein Phosphatase 1

PP1c – PP1 catalytic subunit

RIPPOs – Regulatory Interactors of Protein Phosphatase 1

PP2A – Protein Phosphatase 2A

NEBD – Nuclear envelope breakdown

TMC – Tautomycetin

PB – Polar body

PBE – Polar body emission

IVM – *in vitro* maturation

pH3T3 – phosphorylation of Histone H3 on Threonine 3

pH3S10 – phosphorylation of Histone H3 on Serine 10

NSN – non-surrounded nucleolus

SN – surrounded nucleolus

Abstract

Tightly controlled fluctuations in kinase and phosphatase activity play important roles in regulating M-Phase transitions (e.g., G2/M). Protein Phosphatase 1 (PP1) is one of these phosphatases, with oscillations in activity driving mitotic M-Phase entry, progression, and exit, with evidence from a variety of experimental systems pointing to roles in meiosis as well. Here we report that PP1 is important for M-Phase transitions through mouse oocyte meiosis. Employing a unique small-molecule approach to inhibit or activate PP1 at distinct phases of mouse oocyte meiosis, we found that aberrations in normal cyclical PP1 activity leads to meiotic abnormalities. We report here that temporal control of PP1 activity is essential for G2/M transition, metaphase I/anaphase I transition, and the formation of a normal metaphase II oocyte. Our data also reveal that inappropriate activation of PP1 is more deleterious at G2/M transition than at prometaphase I-to-metaphase I, and that an active pool of PP1 during prometaphase I is vital for metaphase I/anaphase I transition and metaphase II chromosome alignment. Taken together, these results establish that loss of oscillations in PP1 activity causes a range of severe meiotic defects, pointing to essential roles for PP1 in oocytes and female fertility, and more broadly, M-Phase regulation.

Introduction

Accurate oocyte meiosis is essential for female fertility and the development of healthy offspring. Paradoxically, mammalian oocyte meiosis is inherently error-prone, with aneuploidy observed in 10-30% of human oocytes, making aneuploidy a leading cause of miscarriage and birth defects (Hassold and Hunt, 2001; Nagaoka *et al.*, 2012). Key to successful meiosis is precise M-Phase regulation driven by widespread changes in protein phosphorylation (Figure 1). This dynamic phospho-environment is driven by tightly controlled fluctuations in both kinase and phosphatase activity. CDK1 is largely responsible for the increase in protein phosphorylation that propels M-Phase entry and progression (Crncec and Hochegger, 2019). Phosphatases, including the closely related serine/threonine phosphatases Protein Phosphatase 1 (PP1) and Protein Phosphatase 2A (PP2A), regulate M-Phase exit by removing phosphorylations needed to maintain M-Phase and act as the major phosphatases that oppose CDK1 (St-Denis *et al.*, 2016) (Figure 1A-B).

PP1 and PP2A are critically important for mitotic and meiotic M-Phase progression and exit. In mitosis, PP1 and PP2A have unique temporal and spatial regulation, and work in concert to oppose CDK1 activity, preventing M-Phase entry and promoting metaphase-to-anaphase transition (Grallert *et al.*, 2015; Ma *et al.*, 2016; Rogers *et al.*, 2016; Bancroft *et al.*, 2020). In mammalian oocytes, inhibition of PP1/PP2A with reagents such as okadaic acid and Calyculin A causes major meiotic abnormalities, and oocyte-specific conditional loss of PP2A causes substantial defects in chromosome segregation and M-Phase progression (Alexandre *et al.*, 1991; Gavin *et al.*, 1991; Schwartz and Schultz, 1991; Smith *et al.*, 1998; Mailhes *et al.*, 2003; Swain *et al.*, 2003; Wang *et al.*, 2004; Swain *et al.*, 2007; Hu *et al.*, 2014; Tang *et al.*, 2016). However, data also suggest that PP2A is not the only phosphatase that regulates meiosis in mammalian oocytes, and so we sought to elucidate PP1's functions in this important cell type, building on evidence that PP1 has important roles in meiosis in *Drosophila*, *Caenorhabditis elegans*, starfish oocytes, and mammalian spermatogenesis (Oppedisano *et al.*, 2002; Hattersley *et al.*, 2016; Wang *et al.*, 2019a; Swartz *et al.*, 2021).

PP1 is a holoenzyme consisting of a catalytic subunit (PP1c) and one to two regulatory subunits (called PIPs, for PP1-interacting subunit, or RIPPOs, for regulatory interactors of protein phosphatase 1) (Bollen *et al.*, 2010; Heroes *et al.*, 2013). In mammals, there are three PP1c genes and hundreds of RIPPOs, resulting in hundreds of unique holoenzymes with distinctive functions and substrates (Bollen *et al.*, 2010; Heroes *et al.*, 2013). Various forms of PP1 holoenzymes allow PP1 activity to contribute to numerous processes in mitosis, including the G2/M transition, microtubule-kinetochore attachments, the spindle assembly checkpoint, and aspects of cytokinesis (Cheng *et al.*, 2000; Margolis *et al.*, 2003; Winkler *et al.*, 2015; Bhowmick *et al.*, 2019; Capalbo *et al.*, 2019; Conti *et al.*, 2019; Smith *et al.*, 2019; Bancroft *et al.*, 2020). Oscillations in phosphorylation of RIPPOs and PP1c, particularly phosphorylation of PP1c's inhibitory site (pT320 on PP1cα and the equivalent sites in PP1cβ and PP1cγ), during M-Phase play a critical role in the formation of PP1 holoenzymes and PP1 activity, and ultimately M-Phase progression (Dohadwala *et al.*, 1994; Kwon *et al.*, 1997; Nasa *et al.*, 2018). These oscillations in PP1c activity and phosphorylations are observed in mitotic cells, and in starfish and mammalian oocytes (Dohadwala *et al.*, 1994; Kwon *et al.*, 1997; Adhikari *et al.*, 2012; Swartz *et al.*, 2021).

Oocytes present a valuable and unique opportunity to unravel the functions of cell cycle regulatory proteins. A distinctive feature of mammalian oocytes is their relatively slow and synchronous progression through M-Phase of meiosis I during a process called meiotic or oocyte maturation. The G2/M transition to M-Phase exit in mouse oocytes takes approximately 7-10 h (Kalous *et al.*, 2018), whereas the equivalent stage of mitosis in mammalian cell lines lasts 30-60 mins (Kumei *et al.*, 1989; Hesse *et al.*, 2012). Subphases of M-Phase (e.g., metaphase) last hours in oocyte meiosis, versus minutes in mitosis. Additionally, studies of M-Phase in mammalian cultured cells frequently employ chemical or metabolic-induced synchronization to arrest cells at specific mitotic stages, but such methods may induce artifacts (Ly *et al.*, 2015; Ly *et al.*, 2017). Mouse oocytes allow for the study of PP1 at discrete windows of time (e.g., G2/M transition only) without the need for potentially damaging synchronization methods.

As noted above, exposure of mammalian oocytes to okadaic acid or Calyculin A, which inhibit PP1 and PP2A, leads to significant aberrations during *in vitro* meiotic maturation, including accelerated nuclear envelope breakdown (NEBD, G2/M transition), abnormal spindle morphology, meiosis I failure, and aneuploidy (Alexandre *et al.*, 1991; Gavin *et al.*, 1991; Schwartz and Schultz, 1991; Smith *et al.*, 1998; Mailhes *et al.*, 2003; Swain *et al.*, 2003; Wang *et al.*, 2004; Swain *et al.*, 2007). However, only two studies have attempted to tease apart the functions of PP1 and PP2A, finding that PP1 is important for NEBD and chromosome condensation in mouse oocytes (Swain *et al.*, 2003; Swain *et al.*, 2007). To establish the roles that PP1 plays in mammalian oocyte M-Phase, we leveraged a small molecule approach to manipulate PP1 activity in opposition to its anticipated activity (e.g., inappropriately activating PP1 at G2/M) or preventing oscillations in PP1 activity (e.g., inhibiting PP1 for the duration of meiosis). Mouse oocytes were exposed to the PP1 selective inhibitor tautomycetin (Mitsuhashi *et al.*, 2001) and a recently developed PP1 activator PDP-Nal (Wang *et al.*, 2019b) at discrete windows of meiosis (Figure 1C), allowing us to investigate the functions of PP1 in oocytes with unprecedented control. We found that loss of normal PP1 fluctuations caused a range of severe meiotic defects, pointing to roles for PP1 in oocyte M-Phase and female fertility.

Results

<u>PP1 inhibition through meiotic maturation impairs M-Phase exit and reduces oocyte quality.</u>

Prior studies investigating the role of PP1 in mammalian oocytes used inhibitors that inhibit both PP1 and PP2A (e.g., okadaic acid and Calyculin A), which complicates interpretation of data and prevents ascribing effects to one or the other phosphatase. Inhibition of PP1/PP2A arrests oocytes in meiosis I, with oocytes having abnormal spindle morphology and chromosome alignment (Alexandre *et al.*, 1991; Gavin *et al.*, 1991; Schwartz and Schultz, 1991; Mailhes *et al.*, 2003). To assess the functions of PP1 during mouse oocyte meiosis, we utilized the PP1 selective inhibitor tautomycetin (TMC) to reduce PP1 activity. Our first series of studies exposed oocytes to TMC for the duration of *in vitro* maturation (IVM; 0-16 h; Figure 2A), and assessed the ability of oocytes to progress through meiosis I and to metaphase II. TMC impaired M-Phase exit compared to vehicle control, as determined by the presence of a polar body (PB) by phase microscopy (Figure 2B; Veh, 93.6% PB emission (PBE); 0.5 µM TMC 79.2% PBE, $p=0.0216$; 1 µM TMC 95.9% PBE, $p=0.7154$; 2.5 µM TMC 77.6% PBE, $p=0.0119$; 5 µM TMC 70.1% PBE, $p<0.0001$). Using immunofluorescence, we further characterized the meiotic stage of oocytes

and assessed features of oocyte quality. Oocytes were scored as normal or abnormal, with classification as abnormal if a normal metaphase II DNA plate was not present after 16 h of IVM (examples of abnormalities observed in Figure 2D-J'). PP1 inhibition resulted in a substantial increase in meiotic abnormalities in a dose-dependent manner compared to vehicle control (Figure 2C-K; meiotic abnormalities: Veh 10.7% abnormal; 0.5 µM TMC 26.9% abnormal, p=0.1010; 1 µM TMC 16.1% abnormal, p=0.5114; 2.5 µM TMC 34.3% abnormal, p=0.0131; and 5 µM TMC 88.0% abnormal, p<0.0001). These abnormalities ranged from an oocyte being arrested in metaphase I (Figure 2D) to two spindles with no PB (Figure 2G), with increasing doses of TMC leading to more severe defects.

We attempted similar experiments for the duration of IVM with a reagent to manipulate PP1 to an activated state, treating oocytes with the recently developed PP1 activating peptide PDP-Nal (Wang *et al.*, 2019b). PDP-Nal stimulates PP1 activity by uncoupling the PP1 catalytic subunit from RIPPO regulation. However, exposure of oocytes to PDP-Nal for the duration of IVM (0-16 h) resulted in 100% of oocytes dying. Taken together, these data highlight that oscillations in PP1 activity is essential for normal meiotic progression.

<u>Reduced PP1 activity drives G2/M transition in oocytes.</u>

In mitosis, oscillations in PP1 activity are essential for M-Phase progression, with decreased PP1 activity driving G2/M transition and increased PP1 activity being crucial for metaphase/anaphase transition (Moura and Conde, 2019). This cyclic PP1 activity is driven in part by a CDK1-mediated inhibitory phosphorylation of PP1c (pPP1cα(T320) or equivalent sites in PP1cβ and PP1cγ) (Dohadwala *et al.*, 1994; Kwon *et al.*, 1997). The kinetics of PP1 inhibitory phosphorylation follows an identical pattern in starfish and mouse oocytes, suggesting similar fluctuations in PP1 activity during oocyte meiosis (Adhikari *et al.*, 2012; Swartz *et al.*, 2021). Therefore, we sought to determine if a reduction in PP1 activity is critical for meiotic resumption by culturing oocytes in PDP-Nal during the G2/M transition (0-5 h post-meiotic arrest release; Figure 3A). Oocytes were released from prophase I arrest into increasing concentrations of PDP-Nal, the negative control peptide (PDPm-Nal), or standard culture medium (Ctrl). G2/M transition, as determined by NEBD, was hampered by PP1 activation in a dose-dependent manner compared to PDPm-Nal treatment (Figure 3B; NEBD at 5 h: PDPm-Nal 86.7%; Ctrl 86.8%, p >0.9999; 2.5 µM PDP-Nal 74.0%, p=0.0031; 5 µM PDP-Nal 50.3%, p<0.0001; 7.5 µM PDP-Nal 35.7%, p<0.0001; 10 µM PDP-Nal 19.3%, p<0.0001). Moreover, PP1 activation significantly reduced oocyte survival (Figure 3C; Dead oocytes at 5 h: PDPm-Nal 2.3%; Ctrl 0.6%, p=0.2147; 2.5 µM PDP-Nal 12.4%, p=0.0003; 5 µM PDP-Nal 39.5%, p<0.0001; 7.5 µM PDP-Nal 50.0%, p<0.0001; 10 µM PDP-Nal 66.9%, p<0.0001). Critically, the majority of oocyte death was observed in prophase I oocytes, rather than oocytes that had resumed meiosis (Figure 3D; % of oocytes that died at prophase I: 2.5 µM 85.7%; 5 µM 83.5%; 7.5 µM 85.7%; 10 µM 91.7%).

To confirm that PP1 was activated, we investigated the phosphorylation status of two well characterized PP1 substrates, Histone H3 on Threonine 3 (pH3T3) and on Serine 10 (pH3S10). pH3S10 was significantly decreased with 5, 7.5 and 10 µM PDP-Nal treatment compared to PDPm-Nal (Figure 3E; 2.5 µM, 3.2 ± 12% pH3S10 loss, p > 0.9999; 5 µM, 71.1 ± 2.3% pH3S10 loss, p<0.0001; 7.5 µM, 68.2 ± 3.8% pH3S10 loss, p<0.0001; 10 µM, 61.2 ± 1.7% pH3S10 loss, p=0.0001). PP1 activation also caused a reduction of H3T3 phosphorylation on Threonine 3, but

this was only significant with the highest PDP-Nal concentration (10 µM) compared to PDPm-Nal oocytes (Figure 3F; 2.5 µM, 0.9 ± 5.2% pH3T3 loss, p>0.9999; 5 µM, 20.1 ± 6.6% pH3T3 loss, p>0.9999; 7.5 µM, 27.5 ± 1.1% pH3T3 loss, p=0.2299; 10 µM, 88.6 ± 1.0% pH3T3 loss, p<0.0001).

To further define the role of PP1 activity at G2/M transition, we evaluated inappropriate exit from prophase I arrest, in conjunction with TMC treatment. This assay employed a modification of culture conditions used to maintain robust prophase I arrest for extended periods (≥ 16 h), culturing oocytes in 2.5 µM of the phosphodiesterase 3 inhibitor milrinone (Tsafriri *et al.*, 1996; Stein and Schindler, 2011). To evaluate the effects of inhibiting PP1 on the maintenance of prophase I arrest, we used culture conditions that allow a portion of control oocytes to undergo the G2/M transition (NEBD) by culturing oocytes in a low concentration of milrinone (0.2 µM; Figure 4A). With this lower concentration of milrinone (0.2 µM), 36.8% of Veh-treated oocytes were at prophase I arrest after 16 h of culture, compared to 6.2% of IVM control oocytes (no milrinone; p<0.0001). Significantly more TMC-treated oocytes (2.5 µM TMC) underwent the G2/M transition (NEBD) and resumed meiosis as compared to Veh-treated, as reflected by a smaller percentage of oocytes in these treatment groups being at prophase I after 16 h (Figure 4B; Veh 36.8%; 0.5 µM TMC 26.4%, p=0.1826; 1 µM TMC 24.5%, p=0.1288; 2.5 µM TMC 16.2%, p=0.0018; 5 µM TMC 25.7%, p=0.0566). However, despite an increase in G2/M transition with TMC treatment, the percentage of oocytes to exit M-Phase was significantly reduced compared to vehicle control oocytes (Figure 4B; % of oocytes arrested at NEBD at 16 h: Veh 1.3%; 0.5 µM TMC 7.5%, p=0.0399; 1 µM TMC 3.8%, p=0.2753; 2.5 µM TMC 29.7%, p<0.0001; 5 µM TMC 31.6%, p<0.0001).

Additionally, two other effects of TMC treatment in these experiments were observed. First, the chromatin configuration of prophase I oocytes was altered by TMC treatment. Chromatin configuration in prophase I oocytes can be divided into two categories, descriptive of the extent of condensation of the DNA around the nucleolus: non-surrounded nucleolus (NSN) with uncondensed DNA, and surrounded nucleolus (SN) with a ring of condensed DNA around the nucleolus (Figure 4C). Chromatin configuration impacts meiotic competence (ability of an oocyte to reach metaphase II), and developmental potential (ability of a fertilized oocyte to support pre-implantation embryo development), with NSN oocytes displaying lower meiotic competence and developmental potential (Zuccotti *et al.*, 2002; Bellone *et al.*, 2009). Veh-treated oocytes had equal numbers of SN and NSN prophase I oocytes. In contrast, the majority of prophase I oocytes treated with 0.5 µM and 5 µM TMC had NSN configuration (Figure 4C; Veh, 49.1% NSN; 0.5 µM TMC 92.9% NSN, p=0.0049; 1 µM TMC 76.9% NSN, p=0.1199; 2.5 µM TMC 58.3% NSN, p=0.7516; 5 µM TMC 100% NSN, p<0.0001). The second effect of TMC treatment was observed in oocytes that had resumed meiosis. TMC treatment resulted in a dose-dependent increase in meiotic abnormalities, similar to those observed with 16 h TMC treatment under standard IVM conditions (Figure 4D; meiotic abnormalities: Veh 25% abnormal; 0.5 µM TMC 36.7% abnormal, p=0.3208; 1 µM TMC 24% abnormal, p>0.9999; 2.5 µM TMC 69% abnormal, p<0.0001; and 5 µM TMC 97.7% abnormal, p<0.0001). Taken together these results confirm that reducing PP1 activity is central for G2/M transition in oocytes.

<u>PP1 activation during M-Phase reduces oocyte survival and alters chromosome shape.</u>

Having established that inhibition of PP1 is essential for the G2/M transition, we next sought to determine the effects of premature PP1 activation during M-Phase. Oocytes were exposed to increasing concentrations of PDP-Nal from prometaphase (3 h post-meiotic arrest release) to metaphase I (8 h post-meiotic arrest release; Figure 5A), when PP1 activity is low. To confirm that PP1 was activated, we investigated pH3T3 and pH3S10 abundance. At metaphase I (8 h post-meiotic arrest release), pH3T3 fluorescence in oocytes treated with 5 µM, 7.5 µM and 10 µM PDP-Nal was significantly decreased compared to PDPm-Nal-treated oocytes (Figure 5B; 2.5 µM, 24.1 ± 4.7% pH3T3 loss, p= 0.3133; 5 µM, 43.1 ± 4.7% pH3T3 loss, p<0.0001; 7.5 µM, 44.8 ± 5.1% pH3T3 loss, p<0.0001; 10 µM, 46.0 ± 4.9% pH3T3 loss, p<0.0001). A more modest loss of pH3S10 was observed, with 5 and 7.5 µM PDP-Nal treatment significantly different compared to PDPm-Nal (Figure 5C; 2.5 µM, 9.2 ± 4.2% pH3S10 loss, p>0.9999; 5 µM, 26.1 ± 7.8% pH3S10 loss, p=0.0057; 7.5 µM, 45.6 ± 5.0% pH3S10 loss, p<0.0001; 10 µM, 24.0 ± 6.0% pH3S10 loss, p=0.0647). To evaluate if we could achieve a higher extent of PP1 activation (and potentially lower pH3T3 and pH3S10 fluorescence signals at these time points), we tested two higher doses of PDP-Nal, 20 and 40 µM, which are required for sufficient PP1 activation in mitotic cells (Rogers *et al.*, 2016; Wang *et al.*, 2019b). However, treating oocytes with these higher doses of PDP-Nal led to 100% cell death (Figure 5D; Dead oocytes at 8 h: 20 µM PDP-Nal 100% dead, p<0.0001; 40 µM PDP-Nal 100% dead, p<0.0001). Thus, subsequent experiments used PDP-Nal at 2.5, 5.0, 7.5, and 10 µM.

PP1 activation for this five-hour period from prometaphase to metaphase I caused a dose-dependent increase in oocyte death, compared to no death observed in PDPm-Nal-treated oocytes (Figure 5D; % of oocytes dead at 8 h: PDPm-Nal 0%; Ctrl 0%, p>0.9999; 2.5 µM PDP-Nal 3.4%, p=0.0963; 5 µM PDP-Nal 7.5%, p=0.0068; 7.5 µM PDP-Nal 13.3%, p<0.0001; 10 µM PDP-Nal 33.0%, p<0.0001). We also compared the extents of oocyte death observed with 5 h of PDP-Nal treatment from prometaphase to metaphase I and during the G2/M transition (0-5 h post-meiotic arrest release). Surprisingly, PP1 activation for this five-hour period during M-Phase (3-8 h post-meiotic arrest release) was less deleterious than PP1 activation for five hours during the G2/M transition (0-5 h post-meiotic arrest release). Significantly higher numbers of oocytes survived PDP-Nal treatment during M-Phase than during the G2/M transition (Figure 5E; 2.5 µM: 12.4% (0-5 h) vs. 2.9% (3-8 h) dead, p=0.0151; 5 µM: 39.3% (0-5 h) vs. 8.2% (3-8 h) dead, p<0.0001; 7.5 µM: 50% (0-5 h) vs. 13% (3-8 h) dead, p<0.0001;10 µM: 66.9% (0-5 h) vs. 34.9% (3-8 h) dead, p<0.0001).

Finally, various endpoints of oocyte quality were examined in metaphase I oocytes after 5 h of PP1 activation. There was no change in metaphase I plate morphology or distance of the metaphase I plate to the cortex (Supp Figure S1). However, PP1 activation altered the shape of individual chromosomes, with a shift from normal morphology of chromosomes with rounded ends, to chromosomes with pointy ends observed at 10 µM PDP-Nal treatment compared to PDPm-Nal (Figure 5F; Oocytes with at least one pointy chromosome: PDPm-Nal 29.4%; Ctrl 32.2%, p=0.8372; 2.5 µM PDP-Nal 25.0%, p=0.6577; 5 µM PDP-Nal 27.3%, p=0.8322; 7.5 µM PDP-Nal 37.8%, p=0.3964; 10 µM PDP-Nal 58.8%, p=0.0127). Taken together, these results show that reduced PP1 activity during M-Phase plays a key role in oocyte survival and chromosome architecture.

PP1 inhibition during M-Phase slows the timing of meiosis I exit and increases PP1 substrate phosphorylation.

We next sought to determine the impact of PP1 inhibition during M-Phase, given that PP1 activity would be anticipated to be important for meiosis I exit. As our studies above found that TMC treatment for the duration of meiotic maturation impaired meiosis I exit (Figure 2), we decided to treat oocytes with 5 µM TMC from 0 - 7 h or 0 - 9 h post-meiotic arrest release (Figure 6A). Based on analyses of chromatin appearance (Figure 6B) and phosphorylation of the PP1 substrate H3T3 (Figure 6C and Supp Figure S2), the 7-hour timepoint corresponded to late prometaphase / early metaphase I with relatively low global PP1 activity, and the 9-hour timepoint corresponded to the start of M-Phase exit and relatively high global PP1 activity, with a significant loss in pH3T3 detected (Figure 6C, $p<0.0001$).

At 7 h, 100% of Veh control and TMC-treated oocytes were at metaphase I (Veh, n=54 oocytes; TMC, n=50 oocytes). By 9 h, 16% of Veh control oocytes were beginning to exit meiosis I, classified as anaphase I to cytokinesis, whereas only 2.5% TMC-treated oocytes reached this stage of M-Phase exit (Figure 6D; $p=0.0134$). We also examined the metaphase I plate of oocytes for chromosome alignment and distance from the cortex (as illustrated in Supp Figure 3). At 7 h, 60.6% of Veh-treated oocytes and 85.2% of TMC-treated oocytes had an aligned metaphase I plate (just outside of significance at $p=0.0514$). At 9 h, 84.4% of Veh-treated had an aligned metaphase I plate (with this increase from 7 h being statistically significant, $p=0.0463$), and 92.9% of TMC-treated oocytes had an aligned metaphase I plate (Figure 6E; $p=0.2764$ as compared to 7 h). TMC treatment did not appear to affect metaphase spindle migration to the cortex, with no difference in the distance of the metaphase plate to the cortex between the Veh control and TMC-treated groups (Supp Figure S4).

These studies were also used to confirm that TMC treatment results in PP1 inhibition, through characterization of H3T3 phosphorylation. pH3T3 is almost completely lost from chromosomes in oocytes at anaphase I to cytokinesis, and therefore, only metaphase I oocytes were analyzed for pH3T3 signal intensity. (Note: Phosphorylation of H3S10 appears to be stable from NEBD to metaphase II and so was not part of this analysis (Swain *et al.*, 2007; Wang *et al.*, 2016).) In agreement with our other studies (Figure 6C), pH3T3 signal was reduced in control Veh-treated oocytes between 7 and 9 h (Figure 6F; 22.2% loss, $p=0.0113$). This reduction in pH3T3 was not observed with TMC treatment, with no difference in pH3T3 signal in TMC-treated oocytes between 7 and 9 h ($p=0.1793$). Most importantly, there was a significant increase in pH3T3 in TMC-treated oocytes compared to Veh-treated oocytes at both timepoints, suggesting that TMC inhibited PP1-mediated dephosphorylation of H3T3 (Figure 6F; Veh vs. TMC at 7 h, 23.1% pH3T3 increase, $p=0.0332$; Veh vs. TMC at 9 h, 29% pH3T3 increase, $p=0.0002$). Taken together, these data further point to a role for PP1 in M-Phase exit.

Reduced PP1 activity from metaphase I onwards impairs M-Phase exit, but has no detectable effects on oocyte quality.

Our studies noted above (Figure 2K) showed that TMC treatment for the duration of meiotic maturation significantly impaired meiosis I exit, with 22.7% of 5 µM TMC-treated oocytes arrested at metaphase I at 16 h IVM compared to 1.8% of Veh-treated oocytes. Similarly, PP1 inhibition in mitotic cells caused arrest at metaphase, with PP1 found to have essential roles at

the metaphase/anaphase transition (Winkler *et al.*, 2015). To determine if the metaphase I arrest phenotype we observed resulted from a loss of PP1 activity specifically at metaphase I/anaphase I transition, oocytes were treated with TMC in this precise time window from metaphase I onward (8 - 16 h post-meiotic arrest release; Figure 7A). TMC treatment during this time of meiosis reduced M-Phase exit compared to vehicle control, as determined by the presence of a polar body by phase microscopy (Figure 7B; Veh, 100% PBE; 0.5 µM TMC 97.3% PBE, p.0.9999; 1 µM TMC 96.2% PBE, p=0.4955; 2.5 µM TMC 87.6% PBE, p=0.0267; 5 µM TMC 84.7% PBE, p<0.0001). However, the extent of impaired M-Phase exit in these experiments was substantially less than that observed with TMC treatment for the duration of meiosis (0 - 16 h post-meiotic arrest release, Figure 2B). Furthermore, TMC treatment during this time of meiosis did not cause the meiotic abnormalities observed with the longer duration treatment (Figure 7C; meiotic abnormalities: Veh = 15.2% abnormal; 0.5 µM TMC = 8.8% abnormal, p=0.4763; 1 µM TMC = 12.1% abnormal, p>0.9999; 2.5 µM TMC = 22.5% abnormal, p=0.5542; and 5 µM TMC 26.5% abnormal, p=0.3689). These results point to PP1 having critical roles earlier in meiosis.

These surprising results prompted us to hypothesize that loss of PP1 activity specifically during the G2/M transition would have effects later in meiosis, such as on meiotic exit or metaphase II oocyte quality. To test this, oocytes were cultured in TMC during the G2/M transition (0 - 3 h post-meiotic arrest release), then were transferred into medium lacking TMC for the remainder of IVM (3 - 16 h post-meiotic arrest release). However, no difference in meiotic progression was observed in these oocytes, based on analyses of the ability of oocytes to progress through meiosis by phase microscopy and of DNA in DAPI-stained oocytes by fluorescence microscopy (Supp Figure S5).

Discussion

Temporal fluctuations in protein phosphorylations are essential for M-Phase entry and progression. Kinases have a well-established role in this process, however, the contribution and regulation of phosphatases during M-Phase is poorly understood. Here we establish that loss of oscillations in PP1 activity causes a range of severe meiotic defects, pointing to crucial roles for PP1 in oocyte meiosis and, more broadly, M-Phase regulation.

PP1 and chromatin architecture

PP1 plays a key role in regulating chromatin architecture in a variety of systems (Bui *et al.*, 2004; Gil and Vagnarelli, 2019). We found that PP1 inhibition and activation altered chromatin configuration at prophase I and metaphase I, respectively, suggesting that PP1 has important functions in chromatin architecture during oocyte meiosis. Prophase I oocytes have two chromatin configuration states: uncondensed euchromatin (NSN) or a condensed heterochromatin ring that surrounds the nucleolus (SN) (Bogolyubova and Bogolyubov, 2020). PP1 inhibition increased the proportion of prophase I oocytes with NSN chromatin configuration. As oocytes gain the ability to undergo the G2/M transition and exit from prophase I arrest (acquiring an ability known as meiotic competence), there is a shift from NSN to SN chromatin (Zuccotti *et al.*, 2002; Bellone *et al.*, 2009), with this shift accompanied by a translocation of PP1 to the nucleus (Smith *et al.*, 1998; Wang *et al.*, 2004). Post-translational modifications, including phosphorylation of H3, play a major role in heterochromatin formation. In somatic cells,

dephosphorylation of S28 on H3 (H3S28) via the PP1 holoenzyme, PP1:RepoMan, is fundamental for heterochromatin formation (de Castro *et al.*, 2017). H3S28 is phosphorylated in mouse prophase I oocytes, with pH3S28 dephosphorylated in a PP1/PP2A dependent manner at prophase I (Swain *et al.*, 2007). Our findings, taken with these studies, suggest that PP1 plays a role in chromatin configuration changes at the NSN-to-SN transition, potentially via PP1-mediated H3S28 dephosphorylation. Therefore, it is possible that the observed change in chromatin configuration with PP1 inhibition is a product of SN heterochromatin decondensing, however, whether this results in a subsequent loss of meiotic competence is unknown.

Condensins and phosphorylation of H3 play a vital role in metaphase chromosome architecture (Hirota *et al.*, 2004; Fischle *et al.*, 2005; Hirota *et al.*, 2005; Piskadlo *et al.*, 2017). Our data show that PP1 activation reduced M-Phase phosphorylation of two PP1 substrates, H3 at T3 and at S10. During M-Phase, H3 is phosphorylated at T3 by Haspin and at S10 by Aurora B and C (Qian *et al.*, 2011; Balboula and Schindler, 2014; Nguyen *et al.*, 2014; Wang *et al.*, 2016). Phosphorylation of H3 at T3 and S10 appears to play an important role in the recruitment of condensin to chromosomes. In *Drosophila*, Aurora kinases recruit condensin to chromosomes in mitosis, and in mouse oocytes active Haspin is required for Aurora C and condensin chromosome association at metaphase I (Giet and Glover, 2001; Nguyen *et al.*, 2014). Notably, the abnormal pointy chromosome architecture observed with PP1 activation is phenocopied in mouse oocytes with oocyte-specific conditional loss of condensin and Haspin inhibition (Lee *et al.*, 2011; Nguyen *et al.*, 2014). Therefore, activation of PP1, and subsequent reduction of pH3T3 and pH3S10 may lead to loss of chromosome condensin, ultimately causing the observed chromosome abnormalities. Further studies are warranted to determine how PP1 regulates chromosome architecture during M-Phase.

<u>Active pools of PP1 in prometaphase impact M-Phase exit and oocyte quality</u>

In mitosis, PP1 plays a pivotal role in the metaphase/anaphase transition (e.g., silencing the spindle assembly checkpoint and APC/C^{CDC20} activation) and cytokinesis. In human mitotic cells, PP1 inhibition or siRNA-mediated loss of PP1 leads to delayed metaphase/anaphase transition, metaphase arrest, and binucleated cells (a sign of cytokinesis failure) (Cheng *et al.*, 2000; Winkler *et al.*, 2015; Bhowmick *et al.*, 2019; Capalbo *et al.*, 2019; Bancroft *et al.*, 2020). We found a similar phenotype in TMC-treated oocytes, with PP1 inhibition for the duration of meiosis (0-16 h post-meiotic arrest release) causing metaphase I arrest or meiotic abnormalities (e.g., chromosome misalignment at metaphase II or cytokinetic failure (two spindles without a PB)). On the other hand, TMC-treatment from metaphase I-to-metaphase II arrest (8-16 h post-meiotic arrest release) showed remarkably few abnormalities, with oocytes largely able to form a normal metaphase II oocyte by 16 h. These disparate results point to an exciting new role for PP1 during prometaphase that establishes the ability of oocytes to undergo metaphase I/anaphase I transition and form a normal metaphase II oocyte. Approximately 40% of PP1 escapes the CDK1-mediated inhibitory phosphorylation of T320, such that there is a substantial subpopulation of PP1 that could be active to mediate this novel function for PP1 in prometaphase (Olsen *et al.*, 2010). Our data further confirm the presence of an active pool of PP1, with a significant increase in pH3T3 observed in oocytes treated with TMC during prometaphase (0-7 h post-meiotic arrest release).

There are two probable causes of the abnormalities observed in oocytes treated with TMC for the duration of meiosis (0-16 h): (1) continued phosphorylation of prometaphase PP1 substrates, and (2) altered protein stability. Prometaphase PP1 substrates have been revealed in a recent study of meiotic phosphorylation dynamics in starfish oocytes, with a subset of Calyculin A (PP1/PP2A inhibitor) sensitive protein phosphorylations peaking at NEBD and decreasing by metaphase I (Swartz *et al.*, 2021). In oocytes with PP1 inhibition for the duration of meiosis (0-16 h), these early M-Phase PP1 phospho-sites could be maintained, likely contributing to the defects observed in these oocytes. In contrast, in oocytes with PP1 inhibition only during metaphase I (8-16 h), these early M-phase PP1 phospho-sites would not be affected, thus being a possible explanation for the differences in the effects of TMC treatments during these two time windows. Protein phosphorylation, including phosphorylation affected by PP1, also plays a role in proteasomal protein targeting, and therefore protein half-life. PP1-mediated dephosphorylation prevents proteasome-mediated degradation of specific proteins (PER2 and MYC) (Gallego *et al.*, 2006; Dingar *et al.*, 2018). Additionally, PP1-mediated dephosphorylation of CDC20 plays a role in activating APC/C^{CDC20}, the major E3 ligase involved in the degradation of pro-M-Phase proteins at metaphase/anaphase transition (Kim *et al.*, 2017; Bancroft *et al.*, 2020). Degradation of the APC/C^{CDC20} substrates securin and cyclin B starts in prometaphase of oocytes and is essential for metaphase I/anaphase I transition and accurate chromosome segregation (Levasseur *et al.*, 2019; Thomas *et al.*, 2021). Consequently, PP1 inhibition in oocytes at prometaphase may alter the degradation kinetics of proteins, impacting M-Phase progression. Our results show that an active pool of PP1 during prometaphase is essential for M-Phase exit and the formation of normal metaphase II oocytes. The specific PP1 holoenzymes and their substrates involved in these prometaphase events remain to be identified.

<u>PP1 activity is needed to maintain metaphase I chromosome congression</u>

PP1 inhibition during prometaphase (0-7 h post-meiotic arrest release) appears to accelerate chromosome alignment compared to controls. However, this chromosome alignment is lost in oocytes that fail to exit metaphase I by 16 h of maturation. A possible explanation for this observation relates to PP1's kinetochore functions. At the kinetochores, PP1 is in a feedback loop with PP2A-B56. The changing activity of these phosphatases has essential roles in chromosome alignment and microtubule-kinetochore attachments (Vallardi *et al.*, 2017). PP2A-B56 initializes microtubule-kinetochore attachments, aligns chromosomes, and recruits PP1 to the kinetochore, with PP1 then stabilizing microtubule-kinetochore attachments and removing PP2A-B56 from the kinetochore (Liu *et al.*, 2010; Xu *et al.*, 2013; Nijenhuis *et al.*, 2014). Thus, PP1 inhibition may favor kinetochore PP2A-B56 and accelerate chromosome congression initially (as observed at 7 h), but then later, failure to stabilize microtubule-kinetochore attachments could cause chromosome misalignment in metaphase I oocytes at 16 h.

<u>Timing of PP1 activation impacts oocyte survival</u>

As discussed above, the timing of PP1 inhibition significantly impacts M-Phase outcomes in oocytes. Similarly, the sensitivity of oocytes to PP1 activation is dependent on M-Phase stage. PP1 activation at G2/M transition (0-5 h post-meiotic arrest release) reduces oocyte viability significantly more than PP1 activation during M-Phase (3-8 h post-meiotic arrest release). Prophase I arrest (equivalent to G2 in mitosis) is characterized by high phosphatase activity and

low kinase activity, particularly kinases that drive M-Phase (e.g., CDK1) (Swartz *et al.*, 2021). Conversely, high kinase activity and low phosphatase activity maintains M-Phase progression. CDK1 is key to establishing this high kinase / low phosphatase environment, with CDK1-mediated phosphorylations activating other pro-M-Phase kinases and inhibiting phosphatases (Rata *et al.*, 2018; Crncec and Hochegger, 2019; Moura and Conde, 2019). PP1 is part of this kinase/phosphatase interplay; at M-Phase entry, CDK1 phosphorylates PP1 at T320, which inhibits PP1 activity (Dohadwala *et al.*, 1994; Kwon *et al.*, 1997). Conversely, auto-dephosphorylation of T320 reactivates PP1 (Wu *et al.*, 2009). Therefore, it is probable that PP1 activation during M-Phase is less deleterious as compared to the G2/M transition because in M-phase, (a) PP1 has to remove the CDK1-mediated inhibitory phosphorylation of T320, and (b) PP1 has to counter the activity of kinases that have already established high activity. In contrast, at the G2/M transition, high PP1 activity is expected to prevent activation of M-Phase kinases leading to an environment of significantly higher phosphatase activity and lower kinase activity than observed in PDP-Nal-treated oocytes at M-Phase. In support of this hypothesis, PDP-Nal treatment caused a 2-to-2.5-fold greater loss of PP1 substrates pH3T3 and pH3S10 at G2/M transition (0-5 h) compared to M-Phase (3-8 h).

Conclusion

In summary, we found that (a) PP1 has critical roles at multiple stages of mammalian oocyte meiosis, and (b) impairing the anticipated oscillations in PP1 activity negatively impacts M-Phase. Importantly, we show for the first time that the timing of PP1 inhibition/activation significantly influences meiotic outcomes. Our study also highlights the advantage of mouse oocytes for studying PP1 at discrete subphases of M-Phase (e.g., prometaphase vs. metaphase). The extended time frame of M-Phase in mouse oocytes (~10 h from G2/M to M-Phase exit) allows for the investigation of PP1's roles at specific subphases of M-Phase that is not currently possible in M-Phase of mammalian cell lines. Demonstrating the importance of this evaluation of PP1 at specific subphases of M-Phase, our study found a novel role for PP1 in prometaphase that is essential for M-Phase exit. Taken together, our results establish the vital role PP1 plays in mammalian oocyte meiosis, and therefore, female fertility. More broadly, this study underscores the need for accurate phosphatase regulation during M-Phase.

Methods

Animals and Ethics Approval

6-11-week-old female CF-1 mice (Envigo) were used in accordance with Purdue Animal Care and Use Committee guidelines. Mice were housed under a 12 h light: dark cycle with ad libitum water and food.

Oocyte Collection and *In vitro* maturation

Prophase I stage oocytes were collected into Whitten's-HEPES medium (109.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5.5 mM glucose, 0.23 mM pyruvic acid, 4.8 mM lactic acid hemicalcium salt, with 7 mM $NaHCO_3$, 15 mM HEPES) supplemented with 0.25 mM dbcAMP (Sigma-Aldrich, D0627) or 2.5 μM milrinone (Sigma-Aldrich, M4659) to maintain prophase I arrest. Cumulus cells were mechanically removed via repeated pipetting of oocytes before transfer into Whitten's bicarbonate (Whitten's medium without HEPES and with 22 mM

NaHCO3) supplemented with dbcAMP or milrinone. For each experiment, oocytes were pooled from 3-7 females.

For *in vitro* maturation (IVM), oocytes were washed into Whitten's-Bicarb without dbcAMP or milrinone to release oocytes from prophase I arrest. Oocytes were matured for up to 16 h at 37°C, 5% CO_2. For assays to evaluate inappropriate exit from prophase I arrest, oocytes were washed into Whitten's-Bicarb supplemented with 0.2 µM milrinone for 16 h at 37°C, 5% CO_2.

Small molecule inhibitors and activators

Oocytes were cultured in tautomycetin 0.5 - 5 µM (TMC; selective PP1c inhibitor; Bio-Techne, 2305) and 2.5 - 40 µM PDP-Nal (PP1c activator; a gift from Maja Köhn) for the duration of IVM or for discrete windows of time (see Figure 1C). For experiments that added TMC or PDP-Nal during meiosis I (*e.g.,* 3 h post-meiotic arrest release), oocytes still at prophase I arrest at 2.5 h post-dbcAMP/milrinone washout were removed. Finally, for oocytes with TMC treatment for 7 and 9 h post-meiotic arrest release, oocytes still at prophase I arrest were removed 6.5 h post dbcAMP/milrinone washout. Inhibitor/activator concentration and culture conditions for individual experiments are detailed in the results section and figure legends.

Immunofluorescence and F-actin staining

Oocytes were fixed in 4% paraformaldehyde (Sigma-Aldrich, P6148) in HEPES buffer (130 mM KCl, 25 mM HEPES, 3 mM $MgCl_2$, and 0.06% Triton-X, pH 7.4) for 30 minutes at 37°C prior to permeabilization in PBS with 0.1% Triton X-100 (Fisher Scientific, BP151–500) and blocking in PBS with 0.1% BSA (Sigma-Aldrich, A9647) and 0.01% Tween-20 (Sigma-Aldrich, P7949) at room temperature. Oocytes were incubated with primary antibodies for α-tubulin (Zymed, clone Z023, 3605884, 1:100, or Developmental Studies Hybridoma Bank, clone 12G10, 10.6 µg/ml), pH3T3 (Cell Signaling Technology (CST), 13576S, 1:1000), or pH3S10 (CST, 9701S, 1:800) for 16 h at 4°C. After 16 h, oocytes were washed in PBS with 0.1% BSA and 0.1% Tween-20 and incubated with 7.5 µg/ml goat anti-mouse IgG or donkey anti-rabbit conjugated to Alexa Fluor 488 (Jackson ImmunoResearch) at room temperature. For F-actin staining, 140 nM Acti-stain 555 phalloidin (Cytoskeleton Inc., PHDH1-A) was added to the secondary antibody. Oocytes were mounted in VectaShield containing 0.75 µg/ml 4-6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, D9542). Imaging was performed using a Zeiss Axio Observer Z1 microscope with AxioCam MRm Rev3 camera and ApoTome optical sectioning (Carl Zeiss, Inc.). Optical sectioning of oocytes was performed to include all DNA and associated microtubules within the cytoplasm with an optical distance of 1 µm between sections.

Immunofluorescence analysis

Image analysis was performed with ImageJ Fiji distribution (Freeware; National Institutes of Health - https://imagej.nih.gov/ij/). Details for each type of analysis are specified below.

DNA morphology and chromosome alignment: Slides were blinded, and oocytes were imaged and assessed for DNA morphology and chromosome alignment to classify stage of meiosis (*e.g.,* prophase I, metaphase I, metaphase II) and chromosome alignment on the metaphase plate. Oocytes were classified as aligned if all DNA congressed on the metaphase plate, as mild chromosome misalignment if one to two chromosomes had not congressed to the metaphase plate, as moderate misalignment if three to four chromosomes had not congressed to the

metaphase plate, or as severe misalignment if >4 chromosomes had not congressed to the metaphase plate or no metaphase plate was present.

Histone H3 phosphorylation fluorescence intensity (pH3T3 and pH3S10): DNA and histone H3 immunofluorescence optical sections were merged into a single DNA or histone H3 immunofluorescence Z-projection. Image threshold and analyze particles Fiji functions were used to determine DNA region-of-interest on DNA Z-projections automatically. Calculations of the pH3T3 and pH3S10 fluorescence intensity were then performed by first obtaining the value for the background signal (by multiplying the DNA area by the average of the mean background signal), and then subtracting this background signal from the integrated density value of the phospho-Histone H3 signal. Control oocytes were then normalized to an average value of 1, with this used to normalize all other groups. For experiments establishing H3T3 phospho-dynamics through meiosis (Figure 6A), the fluorescence intensity of pH3T3 was performed on all oocytes regardless of their meiotic stage. For all other experiments quantifying pH3T3 and pH3S10 dynamics, only the fluorescence intensity of prometaphase or metaphase I oocytes were measured.

Distance of metaphase I plate to the cortex: A Z-projection of the DNA optical sections and the central F-actin optical section were merged into a single image. The metaphase I spindle migrates to the cortex in a pole-first manner (Supp Figure 3A) (Fabritius *et al.*, 2011). Therefore, a straight line from the center of the metaphase I plate to the closest region of the cortex with this pole-first directionality was measured (Supp Figure 3B).

Statistical Analysis

GraphPad Prism 9.2.0 was used for statistical analysis (GraphPad Software, www.graphpad.com), with a p-value of <0.05 considered statistically significant. For categorical data, the Fisher's exact test was used. For continuous/numerical data, D'Agnostino-Pearson omnibus normality test was performed to determine if the data followed a normal distribution. As all numerical data was not normally distributed, the Kruskal-Wallis test with Dunn's post hoc was used. The exact p-values for each analysis can be found in the results section. Additionally, further information on statistical tests used for each dataset can be found within the figure legends.

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Figure Legends

Figure 1: Phospho-dynamics and M-Phase regulation in oocyte meiosis. (A) Enzymatic activity of CDK1 and PP1 in mouse oocytes from prophase I to metaphase II, a process known as meiotic maturation. PP1 activity is shown as global activity based on the presence of PP1 inhibitory phosphorylation (pT320 on PP1cα). (B) Oocytes are arrested at prophase I (Pro I) until just prior to ovulation. Resumption of meiosis and entry into meiosis I M-Phase is equivalent to the G2/M transition in mitosis. Oocytes progress through nuclear envelope breakdown (NEBD) and metaphase I (Met I), then exit M-Phase. After telophase I (Telo I) completion, oocytes exit meiosis I, but bypass interphase and re-enter M-Phase, arresting at metaphase II (Met II). In mice, it takes ~12 hours to progress from prophase I to metaphase II (at which point oocytes arrest until fertilization), with the time needed to reach each stage noted in the figure. Mass changes in protein phosphorylation drive this M-Phase entry and exit (orange = high phosphorylation, white = low phosphorylation). (C) Experimental windows when PP1 activity is inhibited with tautomycetin (TMC) or activated with PDP-Nal.

Figure 2: PP1 inhibition for the duration of oocyte meiotic maturation reduces meiosis I exit in oocytes and causes meiotic abnormalities. (A) Schematic representation of the experimental design. Prophase I oocytes were cultured in medium lacking meiotic arrest reagents and containing TMC (0.5-5 µM) or vehicle control (0.1% DMSO) for 16 h. (B) Graphical representation of meiotic stage based on phase microscopy at 16 h into culture. Bar graph shows the percentage of oocytes at each meiotic stage. n=48-97 oocytes over 3-5 replicates. * denotes a significant difference from Veh control oocytes (Fisher's exact test). (C-J) Representative images of the oocyte phenotypes observed, apostrophe denotes zoomed-in image, scale bar = 10 µM. Orange dashed line denotes the PB boundary, and orange arrow points to single misaligned chromosomes. (C) Normal metaphase II oocyte. Oocytes that underwent NEBD but had no PB with a normal (D) metaphase I spindle, with (E and E') mild chromosome misalignment, with (F and F') severe chromosome misalignment, or with (G) two spindles. (H) Oocyte at cytokinesis. Metaphase II oocytes, with (I and I') mild chromosome misalignment, or with (J and J') severe chromosome misalignment. Oocytes with DNA ball, ± PB refers to very condensed chromatin in the oocyte, with no obvious meiotic spindle (not shown) (K) Graphical representation of the meiotic stage and DNA morphology of oocytes at 16 h into culture. Solid colors represent normal DNA morphology for the meiotic stage, and hatched colors represent abnormal DNA morphology for the meiotic stage. n=26-75 oocytes over 3-5 replicates. * denotes a significant difference from Veh control oocytes (Fisher's exact test).

Figure 3: PP1 activation impairs meiotic resumption. (A) Schematic representation of the experimental design. Prophase I oocytes were released from meiotic arrest into culture medium containing PDP-Nal (2.5-10 µM), 10 µM PDPm-Nal, or standard culture medium (Ctrl; 0 µM PDP-Nal). NEBD was monitored to track meiotic resumption. (B-C) Graphical representation of the percentage of oocytes to (B) resume meiosis (NEBD) or (C) die over time. n=140-181 oocytes over 7-9 replicates. (D) Graphical representation of meiotic stage at oocytes death; n=21-121 oocytes over 7-9 replicates. (E) Graphical comparison of pH3S10 fluorescence intensity. pH3S10 fluorescence was normalized to expression in Ctrl oocytes. Different letters denote a significant difference between the groups (Kruskal-Wallis test with Dunn's post hoc). n=13-41 oocytes over 2 replicates. Representative images of oocyte pH3S10 fluorescence,

scale = 10 µM. (F) Graphical comparison of pH3T3 fluorescence intensity. pH3T3 fluorescence was normalized to expression in Ctrl oocytes. Different letters denote a significant difference between the groups (Kruskal-Wallis test with Dunn's post hoc). n=18-56 oocytes over 3-4 replicates. Representative images of oocyte pH3T3 fluorescence, scale = 10 µM. Scatter dot plots show each individual oocyte fluorescence intensity plus mean and SEM.

Figure 4: PP1 inhibition enhances NEBD, reduces M-Phase exit, and causes meiotic abnormalities in oocytes in culture conditions that allow partial maintenance of prophase I arrest. (A) Schematic representation of the experimental design. Prophase I oocytes were washed into medium containing 0.2 µM milrinone and increasing amounts of TMC (0.5-5 µM) or vehicle control (0.1% DMSO) for 16 h; under these culture conditions, ~37% of oocytes will undergo the G2/M transition, exiting from prophase I arrest. (B) Graphical representation of meiotic stage based on phase microscopy at 16 h into culture. IVM Ctrl oocytes were cultured in milrinone-free medium. Bar graph shows the percentage of oocytes at each meiotic stage. n=53-152 oocytes over 3-6 replicates. * denotes a significant difference from Veh control oocytes (Fisher's exact test). (C) Graphical representation and images of DNA morphology in prophase I oocytes at 16 h into culture. Orange arrow points to the nucleolus and scale bar = 10 µM. n=12-55 oocytes over 3-6 replicates. * denotes a significant difference from Veh control oocytes (Fisher's exact test). (D) Graphical representation of the meiotic stage and DNA morphology of oocytes at 16 h into culture. Solid colors represent normal DNA morphology for the meiotic stage, and hatched colors represent abnormal DNA morphology for the meiotic stage. n=25-86 oocytes over 3-6 replicates. * denotes a significant difference from Veh control oocytes (Fisher's exact test).

Figure 5: PP1 activation from prometaphase to metaphase of meiosis I decreases oocyte survival and alters chromosome morphology. (A) Schematic representation of the experimental design. Prophase I oocytes were released from meiotic arrest and cultured for 3 h before treatment with increasing concentrations of PDP-Nal (2.5-40 µM), PDPm-Nal (10 µM) or standard culture medium (Ctrl; 0 µM PDP-Nal). (B) Graphical comparison of pH3T3 fluorescence intensity. pH3T3 fluorescence was normalized to expression in Ctrl oocytes. Different letters denote a significant difference between the groups (Kruskal-Wallis test with Dunn's post hoc). n=22-41 oocytes over three replicates. Representative images of oocyte pH3T3 fluorescence, scale = 10 µM. (C) Graphical comparison of pH3S10 fluorescence intensity. pH3S10 fluorescence was normalized to expression in Ctrl oocytes. Different letters denote a significant difference between the groups (Kruskal-Wallis test with Dunn's post hoc). n=28-48 oocytes over three replicates. Representative images of oocyte pH3S10 fluorescence, scale = 10 µM. (D) Graphical representation of the percentage of oocyte death over time. n=29-106 oocytes over 2-5 replicates. (E) Graphical representation of percentage of oocytes dead with 5 h of PDP-Nal treatment at G/2M (0-5 h; solid color) or during meiosis I (3-8 h; hashed color). * denotes a significant difference between 0-5 h and 3-8 h PDP-Nal treated oocytes (Fisher's exact test) (F) Graphical representation of the percentage of oocytes with at least one pointy chromosome, with sample images of DAPI staining showing chromosome morphology. n=34-59 oocytes over five replicates. * denotes a significant difference from PDPm-Nal control oocytes (Fisher's exact test). Scatter dot plots show each individual oocyte fluorescence intensity plus mean and SEM.

Figure 6: PP1 inhibition during meiosis I impairs M-Phase exit and increases PP1 substrate phosphorylation. (A) Schematic representation of the experimental design. Prophase I oocytes were released from meiotic arrest and cultured in medium containing TMC (5 µM) or vehicle control (0.1% DMSO) for 7 or 9 h. (B) Graphical representation of meiotic stages in control untreated oocytes at 7, 8, 9, and 10 h post-meiotic arrest release. n=35-38 oocytes over two replicates. (C) Graphical comparison of pH3T3 fluorescence intensity over time in untreated oocytes. pH3T3 fluorescence was normalized to expression at 7 h post-meiotic arrest release. Different letters denote a significant difference between the groups (Kruskal-Wallis test with Dunn's post hoc). n=35-38 oocytes over two replicates. (D) Graphical representation of stages of meiotic exit at 9 h post-meiotic arrest release. * denotes a significant difference (Fisher's exact test). n=50-79 oocytes over two replicates. (E) Graphical representation of chromosome alignment in metaphase I oocytes at 7 or 9 h post-meiotic arrest release. * denotes a significant difference (Fisher's exact test). n=27-56 oocytes over two replicates. (E) Graphical comparison of pH3T3 fluorescence intensity. pH3T3 fluorescence was normalized to expression in Veh oocytes at 7 h. Different letters denote a significant difference between the groups (Kruskal-Wallis test with Dunn's post hoc). n=42-77 oocytes over two replicates. Representative images of oocyte pH3T3 fluorescence, scale = 10 µM. Line graph shows mean and SEM of pH3T3 fluorescence every hour. Scatter dot plots show each individual oocyte fluorescence intensity plus mean and SEM.

Figure 7: PP1 inhibition from metaphase I onwards impairs meiosis I exit, but has no impact on meiotic abnormalities. (A) Schematic representation of the experimental design. Prophase I oocytes were released from meiotic arrest into standard culture medium for 8 h before being cultured in medium containing TMC (0.5-5 µM) or vehicle control (0.1% DMSO). (B) Graphical representation of meiotic stage based on phase microscopy at 16 h into culture. Bar graph shows the percentage of oocytes at each meiotic stage. n=50-52 oocytes over two replicates. * denotes a significant difference from Veh control oocytes (Fisher's exact test). (C) Graphical representation of the meiotic stage and DNA morphology of oocytes at 16 h into culture. Solid colors represent normal DNA morphology for the meiotic stage, and hatched colors represent abnormal DNA morphology for the meiotic stage. n=33-40 oocytes over two replicates.

Supp Figure 1: PP1 activation from prometaphase to metaphase of meiosis I does not impact metaphase I plate position or chromosome alignment. (A) Schematic representation of the experimental design. Prophase I oocytes were released from meiotic arrest and cultured for 3 h before treatment with increasing concentrations of PDP-Nal (2.5-10 µM), PDPm-Nal (10 µM) or standard culture medium (Ctrl; 0 µM PDP-Nal). (B) Graphical comparison of metaphase plate distance to oocyte cortex (Kruskal-Wallis test with Dunn's post hoc). n=34-60 oocytes over 4-5 replicates. Scatter dot plots show each individual oocytes metaphase plate distance to cortex plus mean and SEM. (C) Graphical representation of chromosome alignment in metaphase I oocytes (Fisher's exact test). n=43-69 oocytes over 4-5 replicates.

Supp Figure 2: pH3T3 expression at specific stages of meiotic maturation. Representative images of oocyte pH3T3 fluorescence, DNA and DIC at metaphase I (Met I; 7-8 h), anaphase I/telophase I (ana I/telo I; 9-10 h), cytokinesis, and early metaphase II (met II forming; 10h).

Time denotes the number of hours into meiotic maturation that individual stages of meiosis were observed. Orange dashed line denotes insert region, scale = 10 µM.

Supp Figure 3: Metaphase I plate to cortex measurement. (A) Schematic representation of the metaphase I spindle migration during meiosis I. Purple dashed arrow denotes pole-first directionality of the spindle migration. (B) Representative image of metaphase I plate to cortex measurement. Orange dashed line denotes distance measured, scale = 10 µM.

Supp Figure 4: PP1 inhibition during meiosis I does not impact metaphase I plate position. (A) Schematic representation of the experimental design. Prophase I oocytes were released from meiotic arrest and cultured in medium containing TMC (5 µM) or vehicle control (0.1% DMSO) for 7 or 9 h. (B) Graphical comparison of metaphase I plate distance to oocyte cortex at 7 or 9 h post-meiotic arrest release (Kruskal-Wallis test with Dunn's post hoc). n=27-55 oocytes over two replicates. Scatter dot plots show each individual oocytes metaphase plate I distance to cortex plus mean and SEM.

Supp Figure 5: PP1 inhibition at G2/M transition only does not cause significant meiotic abnormalities. (A) Schematic representation of the experimental design. Prophase I oocytes were released from meiotic arrest and cultured for 3 h in medium containing TMC (0.5-5 µM) or vehicle control (0.1% DMSO) before being washed into standard culture medium (no TMC or DMSO). (B) Graphical representation of meiotic stage based on phase microscopy at 16 h into culture. Bar graph shows the percentage of oocytes at each meiotic stage. n=47-49 oocytes over two replicates. (C) Graphical representation of the meiotic stage and DNA morphology of DAPI-stained oocytes at 16 h into culture. See Figure 2 for examples and descriptions. Solid colors represent normal DNA morphology for the meiotic stage, and hatched colors represent abnormal DNA morphology for the meiotic stage. n=27-36 oocytes over two replicates.

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Figure 1

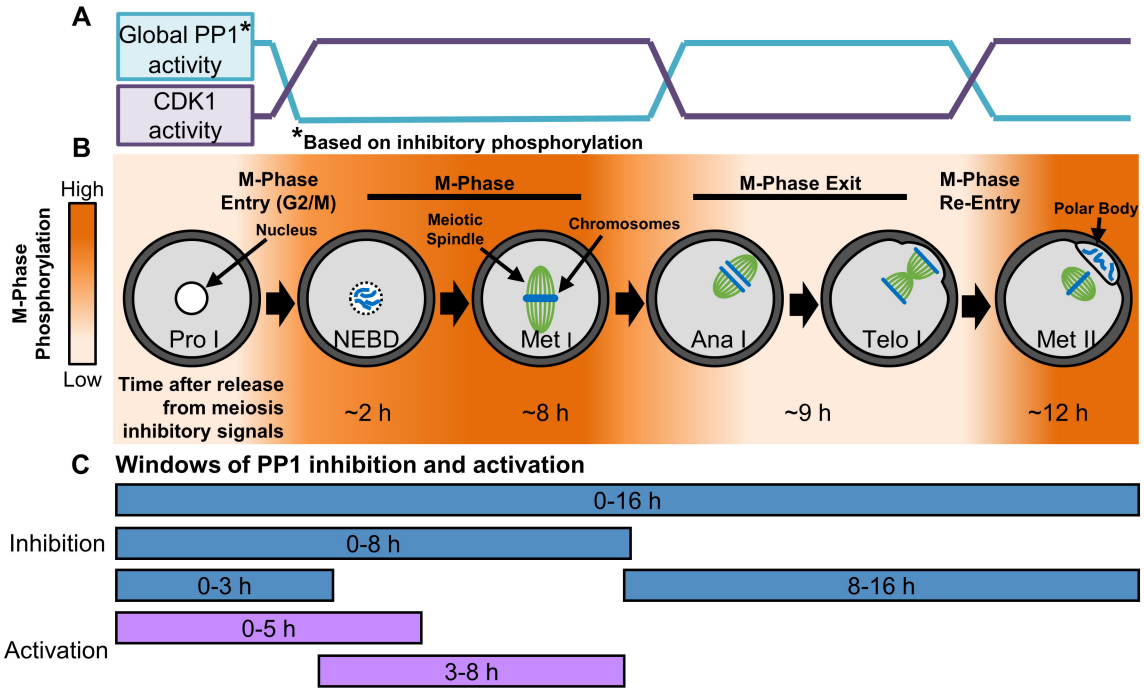

A
Global PP1* activity
CDK1 activity
*Based on inhibitory phosphorylation
B
High
M-Phase Phosphorylation
Low
M-Phase Entry (G2/M)
M-Phase
M-Phase Exit
M-Phase Re-Entry
Nucleus
Meiotic Spindle
Chromosomes
Polar Body
Pro I
NEBD
Met I
Ana I
Telo I
Met II
Time after release from meiosis inhibitory signals
~2 h
~8 h
~9 h
~12 h
C
Windows of PP1 inhibition and activation
Inhibition
0-16 h
0-8 h
0-3 h
8-16 h
Activation
0-5 h
3-8 h

Figure 2

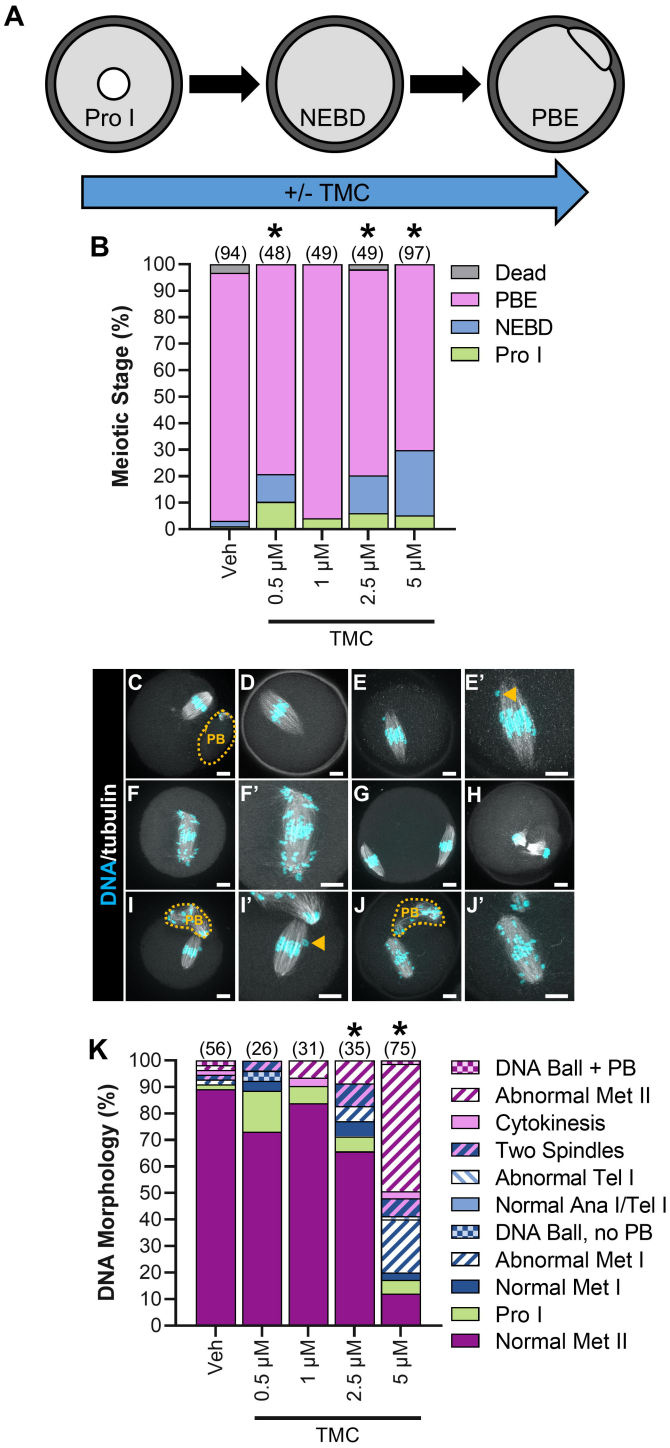

A
Pro I
NEBD
PBE
+/- TMC
B
Meiotic Stage (%)
(94) (48) (49) (49) (97)
Dead
PBE
NEBD
Pro I
Veh
0.5 μM
1 μM
2.5 μM
5 μM
TMC
DNA/tubulin
PB
K
DNA Morphology (%)
(56) (26) (31) (35) (75)
DNA Ball + PB
Abnormal Met II
Cytokinesis
Two Spindles
Abnormal Tel I
Normal Ana I/Tel I
DNA Ball, no PB
Abnormal Met I
Normal Met I
Pro I
Normal Met II

Figure 3

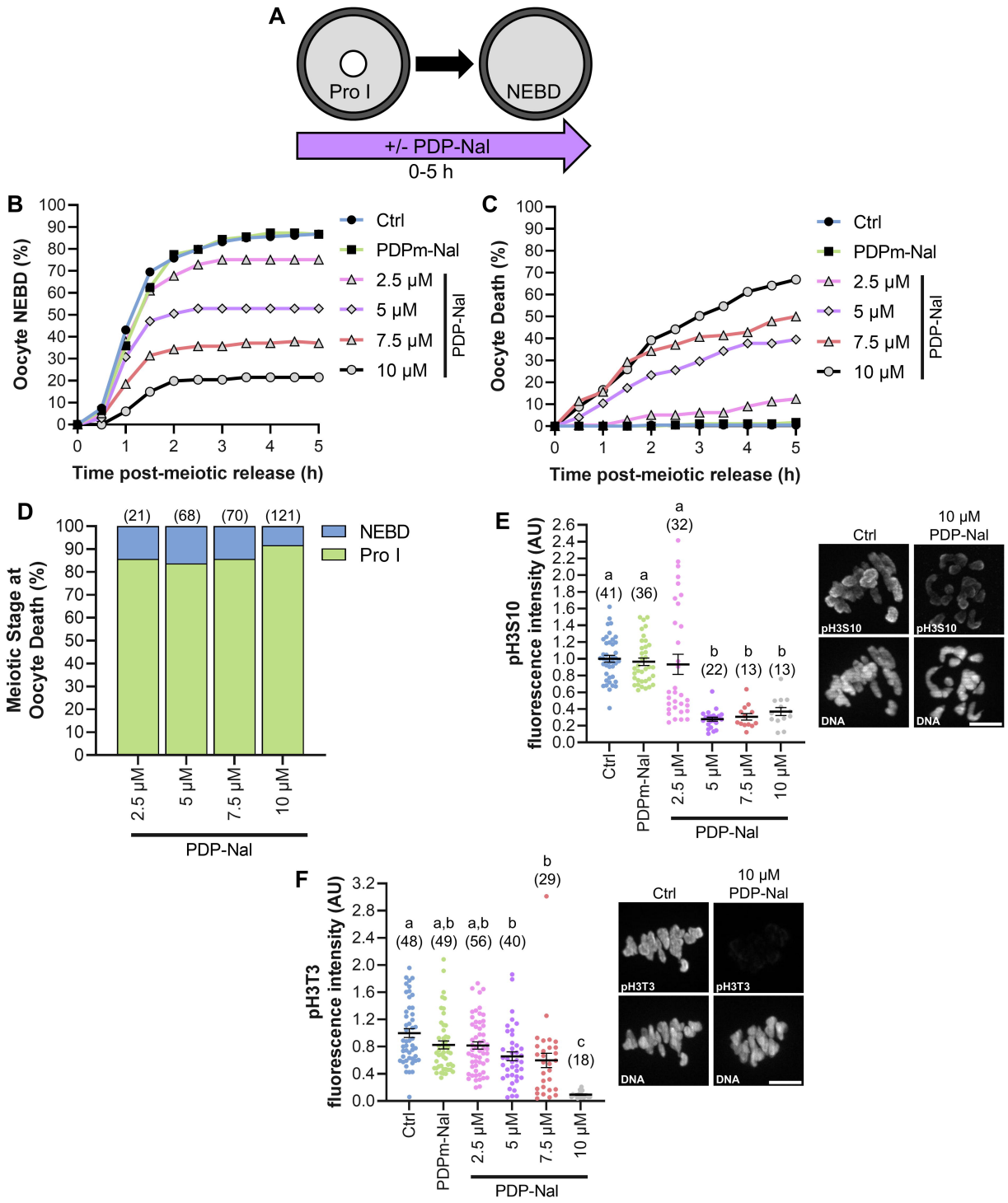

A
Pro I
NEBD
+/- PDP-Nal
0-5 h
B
Oocyte NEBD (%)
Time post-meiotic release (h)
Ctrl
PDPm-Nal
2.5 μM
5 μM
7.5 μM
10 μM
PDP-Nal
C
Oocyte Death (%)
Time post-meiotic release (h)
D
Meiotic Stage at Oocyte Death (%)
(21) (68) (70) (121)
NEBD
Pro I
2.5 μM
5 μM
7.5 μM
10 μM
PDP-Nal
E
pH3S10 fluorescence intensity (AU)
a (41) a (36) a (32) b (22) b (13) b (13)
Ctrl
PDPm-Nal
2.5 μM
5 μM
7.5 μM
10 μM
PDP-Nal
Ctrl
10 μM PDP-Nal
pH3S10
DNA
F
pH3T3 fluorescence intensity (AU)
a (48) a,b (49) a,b (56) b (40) b (29) c (18)
Ctrl
PDPm-Nal
2.5 μM
5 μM
7.5 μM
10 μM
PDP-Nal
Ctrl
10 μM PDP-Nal
pH3T3
DNA

Figure 4

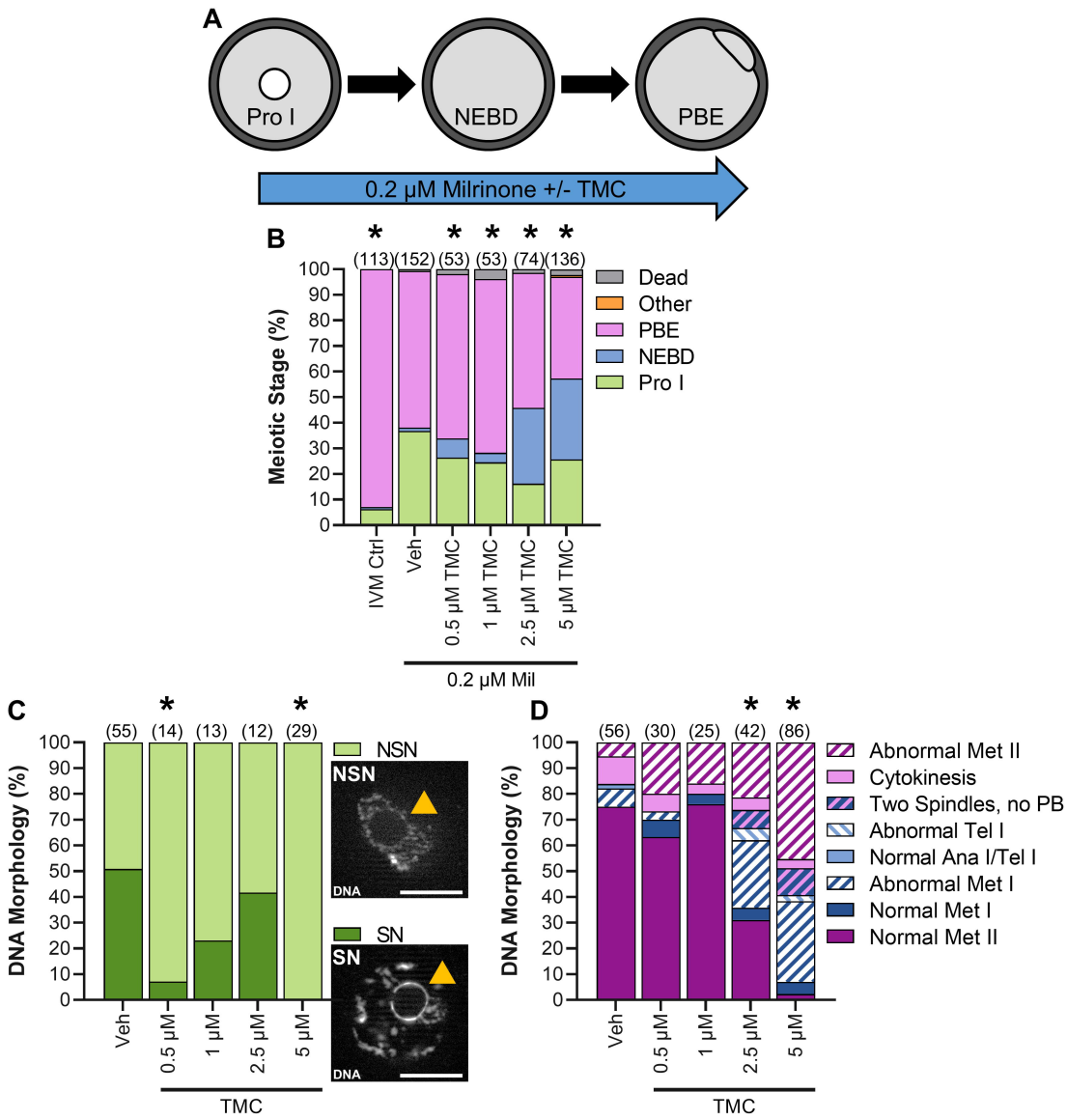

A
Pro I
NEBD
PBE
0.2 μM Milrinone +/- TMC
B
Meiotic Stage (%)
(113)(152)(53)(53)(74)(136)
Dead
Other
PBE
NEBD
Pro I
IVM Ctrl
Veh
0.5 μM TMC
1 μM TMC
2.5 μM TMC
5 μM TMC
0.2 μM Mil
C
DNA Morphology (%)
(55) (14) (13) (12) (29)
NSN
SN
DNA
Veh
0.5 μM
1 μM
2.5 μM
5 μM
TMC
D
DNA Morphology (%)
(56) (30) (25) (42) (86)
Abnormal Met II
Cytokinesis
Two Spindles, no PB
Abnormal Tel I
Normal Ana I/Tel I
Abnormal Met I
Normal Met I
Normal Met II
Veh
0.5 μM
1 μM
2.5 μM
5 μM
TMC

Figure 5

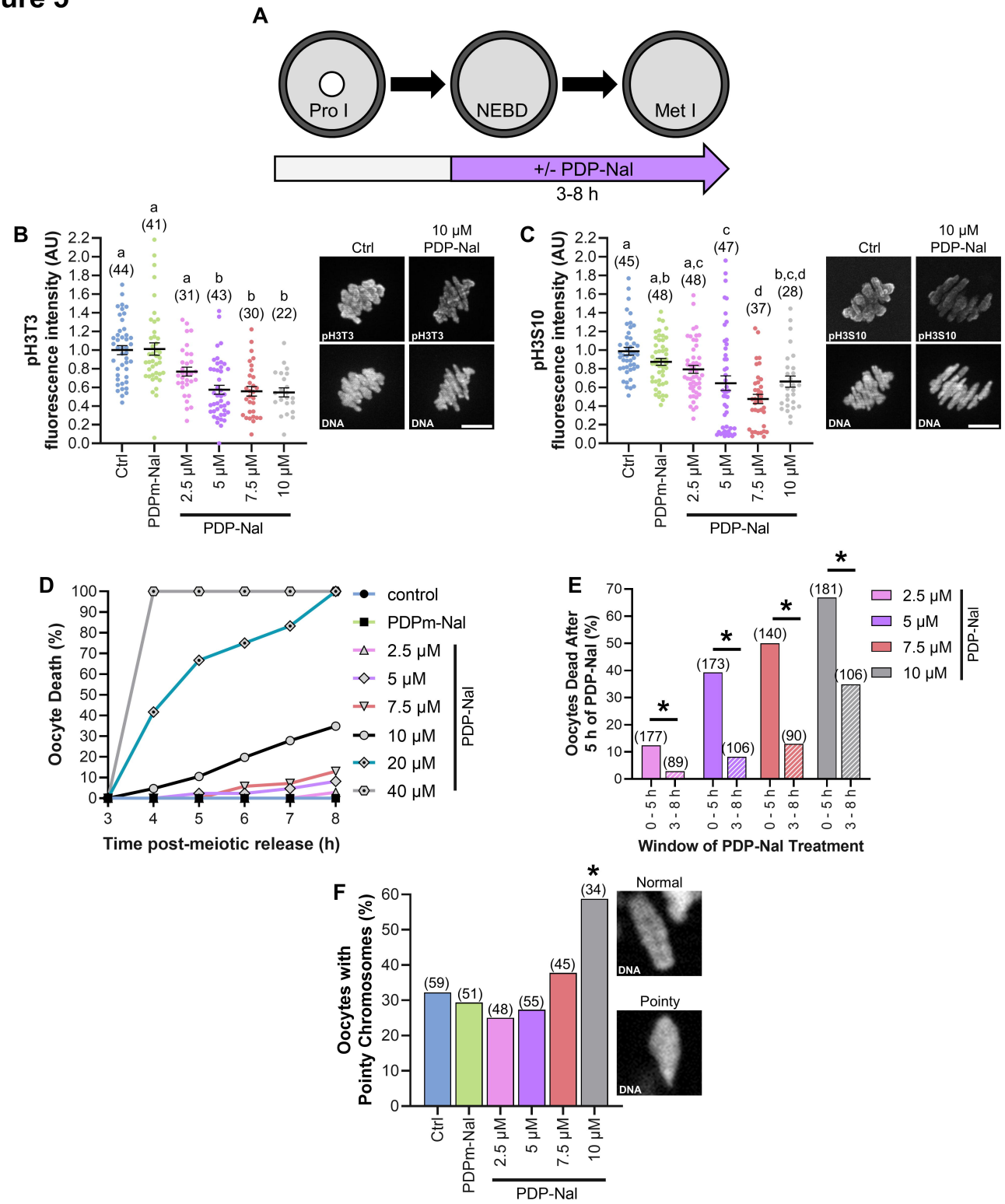

A
Pro I
NEBD
Met I
+/- PDP-Nal
3-8 h
B
pH3T3 fluorescence intensity (AU)
Ctrl
PDPm-Nal
2.5 µM
5 µM
7.5 µM
10 µM
PDP-Nal
10 µM PDP-Nal
pH3T3
DNA
C
pH3S10 fluorescence intensity (AU)
pH3S10
D
Oocyte Death (%)
Time post-meiotic release (h)
control
PDPm-Nal
20 µM
40 µM
E
Oocytes Dead After 5 h of PDP-Nal (%)
Window of PDP-Nal Treatment
0 - 5 h
3 - 8 h
F
Oocytes with Pointy Chromosomes (%)
Normal
Pointy

Figure 6

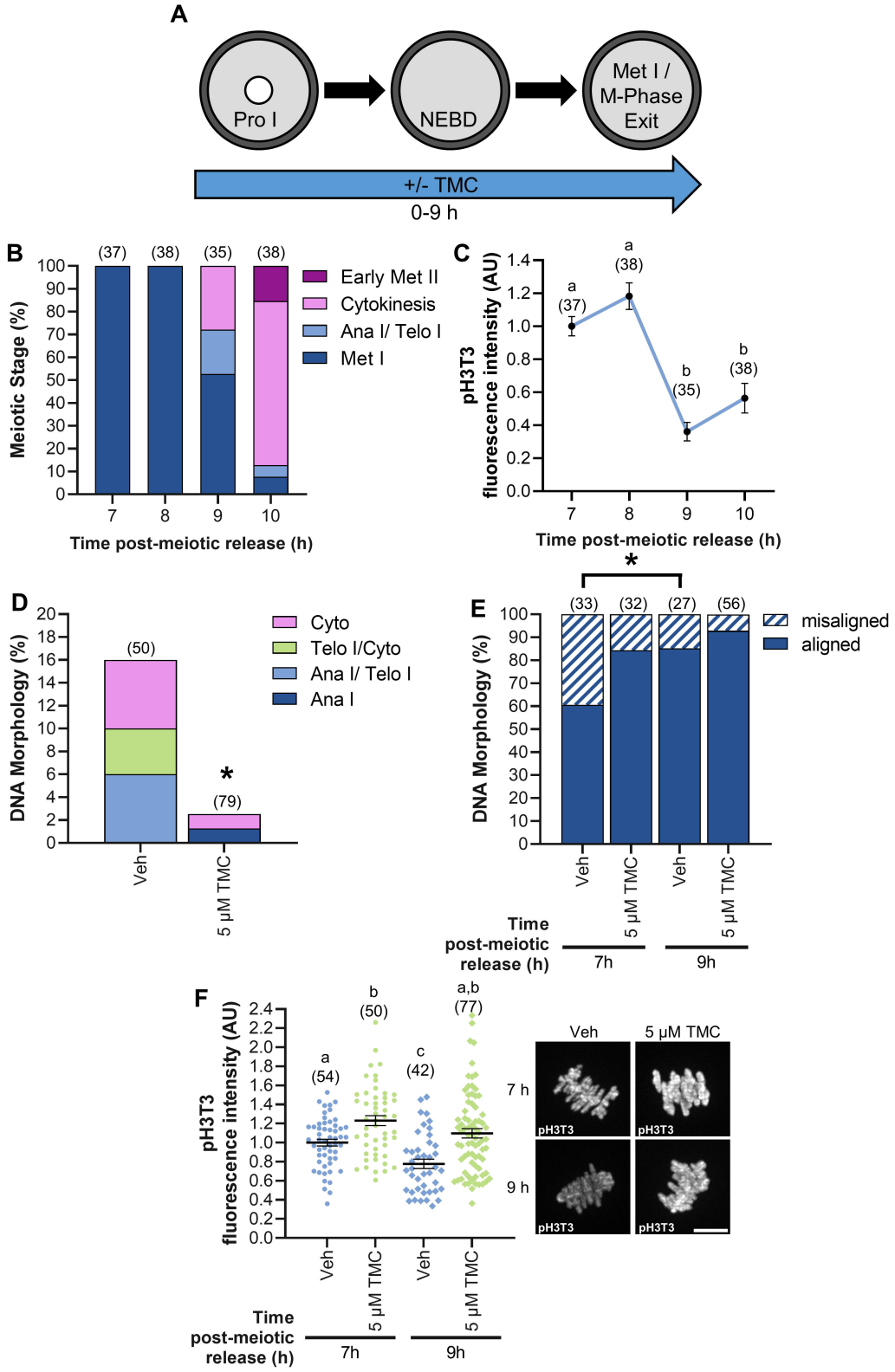
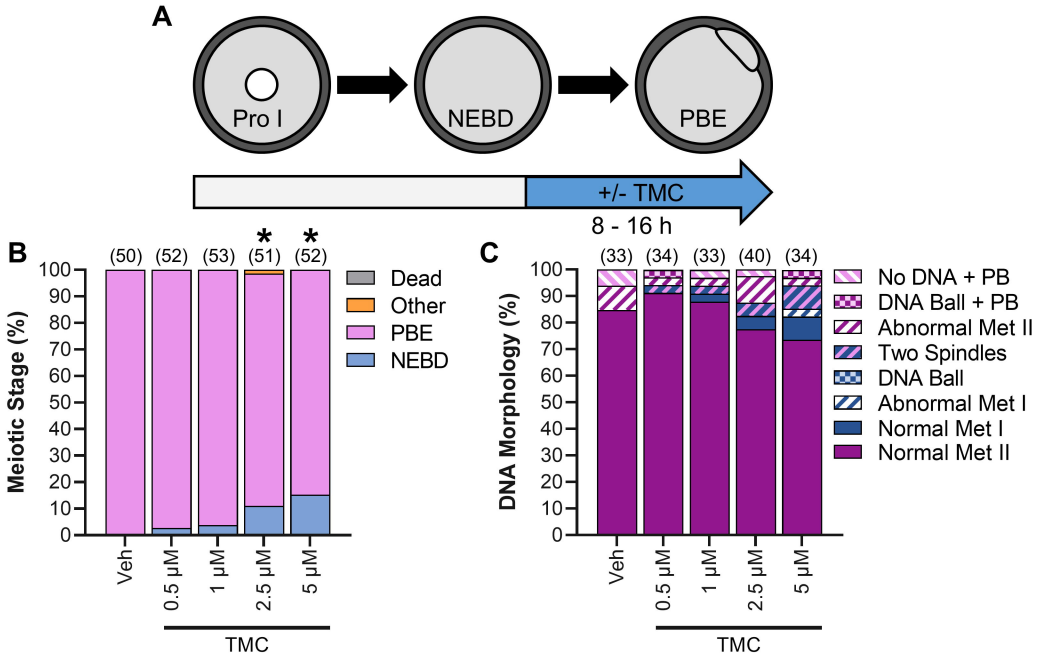

A
Pro I
NEBD
Met I / M-Phase Exit
+/- TMC
0-9 h
B
Meiotic Stage (%)
Time post-meiotic release (h)
Early Met II
Cytokinesis
Ana I/ Telo I
Met I
(37)
(38)
(35)
(38)
C
pH3T3 fluorescence intensity (AU)
Time post-meiotic release (h)
a (37)
a (38)
b (35)
b (38)
D
DNA Morphology (%)
Cyto
Telo I/Cyto
Ana I/ Telo I
Ana I
(50)
(79)
*
Veh
5 μM TMC
E
DNA Morphology (%)
misaligned
aligned
(33)
(32)
(27)
(56)
*
Veh
5 μM TMC
Veh
5 μM TMC
Time post-meiotic release (h)
7h
9h
F
pH3T3 fluorescence intensity (AU)
a (54)
b (50)
c (42)
a,b (77)
Veh
5 μM TMC
Veh
5 μM TMC
Time post-meiotic release (h)
7h
9h
Veh
5 μM TMC
7 h
9 h
pH3T3

Figure 7


A
Pro I
NEBD
PBE
+/- TMC
8 - 16 h
B
Meiotic Stage (%)
(50) (52) (53) (51) (52)
*
*
Dead
Other
PBE
NEBD
Veh
0.5 μM
1 μM
2.5 μM
5 μM
TMC
C
DNA Morphology (%)
(33) (34) (33) (40) (34)
No DNA + PB
DNA Ball + PB
Abnormal Met II
Two Spindles
DNA Ball
Abnormal Met I
Normal Met I
Normal Met II
Veh
0.5 μM
1 μM
2.5 μM
5 μM
TMC