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Supplemental Information

**The Phosphatase Dusp7 Drives Meiotic Resumption
and Chromosome Alignment in Mouse Oocytes**

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Supplemental Files for

Title:

**The phosphatase Dusp7 drives meiotic resumption and chromosome
alignment in mouse oocytes**

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Supplemental Experimental Procedures

Vectors

eGFP-DUSP7 as well as H2B-mRFP and eGFP-MAP4 constructs were described before (Pfender et al., 2015; Schuh and Ellenberg, 2007). Cdk1, CyclinB1, CDC25B and MAD1 were amplified from cDNA and inserted into pGEMHE vectors with the corresponding tags. cPKC constructs were obtained from I. Vallis (MRC Laboratory of Molecular Biology, Cambridge, UK) and cloned into pGEMHE for mRNA preparation. HA-MAPK in pCDNA3.1 was from Addgene (8974) (Dimitri et al., 2005). For immunoprecipitation experiments with DUSP7 the eGFP-tag in pEGFP was replaced by a Flag-tag. For bacterial expression DUSP7 was cloned via Gibson Assembly (New England Biolabs) into pET28a(+). To create point mutants the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) was used. In DUSP7, cysteine 333 was replaced with serine (C333S) to create the catalytically inactive mutant. In the MAPK binding mutant arginine 104 and 105 were replaced by methionine (R104/105M). Similarly, threonine 14 and tyrosine 15 in Cdk1 were replaced by alanine and phenylalanine, respectively, to obtain Cdk1^{AF}.

Immunoprecipitation of Flag-DUSP7 and HA-MAPK from HeLa cells

HeLa cells were plated on 10 cm plates and transfected using Fugene 6 (Promega) according to manufacturer's instructions. Expression was performed for 48h and cells were removed from the plates by trypsin. Subsequently, they were washed two times with PBS and resuspended in lysis buffer (Tanoue et al., 2002). Immunoprecipitation with M2-Flag-agarose (Sigma) was performed as described by the manufacturer,

except that lysis buffer was used for washing. Input and immunoprecipitated samples were analyzed by SDS PAGE and immunoblotting.

Quantitative RT-PCR

Total RNA was isolated from at least ten oocytes with the RNeasy Mirco Plus Kit (Qiagen) and subsequently used for a reverse transcription PCR with the High Capacity RNA to cDNA Kit (Applied Biosystems). Quantitative real time PCR was performed with the SYBR green master mix (Applied Biosystems) on a ViiA 7 Real-Time PCR System (Applied Biosystems) according to the manufactures instructions. The oligo pairs used for GAPDH were 5'-AGAGCTGAACGGGAAGCTCACT-3' and 5'-TGCCTGCTTCACCACCTTCTTGAT-3', for DUSP7 5'-TCACCTACAAGCAAATCCCC-3' and 5'-TGTCACTGAACGGCTGATG-3', and for PKC β 5'-TTAACTTCCTGATGGTGCTGG-3' and 5'-GAGTTCATCTGTACCCTTCCG-3'.

Purification of His-DUSP7 from Bacteria

His-tagged DUSP7 was expressed in *E.coli* strain BL21(DE3) overnight at 18 °C. Bacteria were pelleted the next morning and washed once with PBS. They were taken up in IMAC 5 buffer (20 mM Tris pH 8, 300 mM NaCl, 5 mM Imidazole, complete protease inhibitors (Roche)) and lysed using an EmulsiFlex C5. After centrifugation for 20 minutes at 25.000 g the supernatant was added on Ni-IDA agarose (Macherey and Nagel) and incubated for at least 3 h at 4 °C to allow binding. Subsequently, the beads were washed once in IMAC 20 buffer (see IMAC 5, but 20 mM Imidazole instead), once in IMAC 80 buffer + 1 mM MgATP + 0.1% Triton X-100, twice in

IMAC 80 buffer. Elution was performed on a small column with one bead volume of IMAC 500 buffer.

The purity of the purified protein was analyzed by SDS PAGE and Instant Blue (Expedeon) staining. The activity was determined by a para-Nitrophenyl Phosphate (pNPP, New England Biolabs) assay (Dowd et al., 1998).

In vitro phospho-peptide screening assay and generation of the Venn diagram

Screening plates containing the synthesized peptides were purchased from JPT (product codes PhSS-Y-360-250 and PhSS-360-250). The same assay buffer as for the pNPP assay was used for the screening and the assay was incubated for 2 h at 37 °C. The reaction was stopped with Biomol green solution (Enzo) and incubated for 15 min at room temperature to allow color development. A Tecan F200 plate reader was used to analyze the absorbance at 620 nm. For each assay, Z^* -scores were calculated individually and a value greater than two was considered significant (Zhang, 2011). All potential hits were used as input to create a Venn diagram with help of the website <http://www.interactivenn.net/> (Heberle et al., 2015). The list of hits including all reads and the Z^* -scores is available as Supplemental File S5. The interactive Venn diagram can be recreated using Supplemental File S6 as input on the above mentioned website.

Histone H1 kinase assay

All oocytes were inspected using a dissection stereo microscope every 30 minutes. The first oocytes that performed NEBD were grouped together and left aside. They were taken as the last sample (6h post NEBD). The same procedure was performed

for the 5h time point. This ensures that only oocytes that underwent NEBD were used for the later time points. These are also the time points which were analyzed for Cdk1 kinase activity in Figure 3 B. We used the same procedure for all Cdk1 kinase assays that are shown in the manuscript. Three mouse oocytes in the desired stage of development were transferred into a reaction tube and snap frozen in liquid nitrogen. To thaw them, 2.5 μ L of two-fold concentrated H1 oocyte lysis buffer (160 mM beta-glycero-phosphate, 40 mM EGTA, 30 mM $MgCl_2$, 2 mM DTT, 2x EDTA-free complete protease inhibitors (Roche)) was added and the oocytes were lysed by vortexing and centrifugation at 20.000 rpm, 4 $^{\circ}C$. By addition of 2.5 μ L two-fold concentrated H1 solution (2 mg/mL purified Histon H1 (Millipore), 0.5 mCi/mL gamma- ^{33}P -ATP) the reactions were started and incubated at 30 $^{\circ}C$ for 30 min. The reactions were stopped by adding 5 μ L two-fold concentrated laemmli sample buffer and heated at 95 $^{\circ}C$ for 5 min. Samples were separated via SDS-PAGE and the gel was stained with Instant Blue (Expedeon). After drying on a sheet of Whatman paper, incorporation of ^{33}P was analyzed by autoradiography.

Supplemental References

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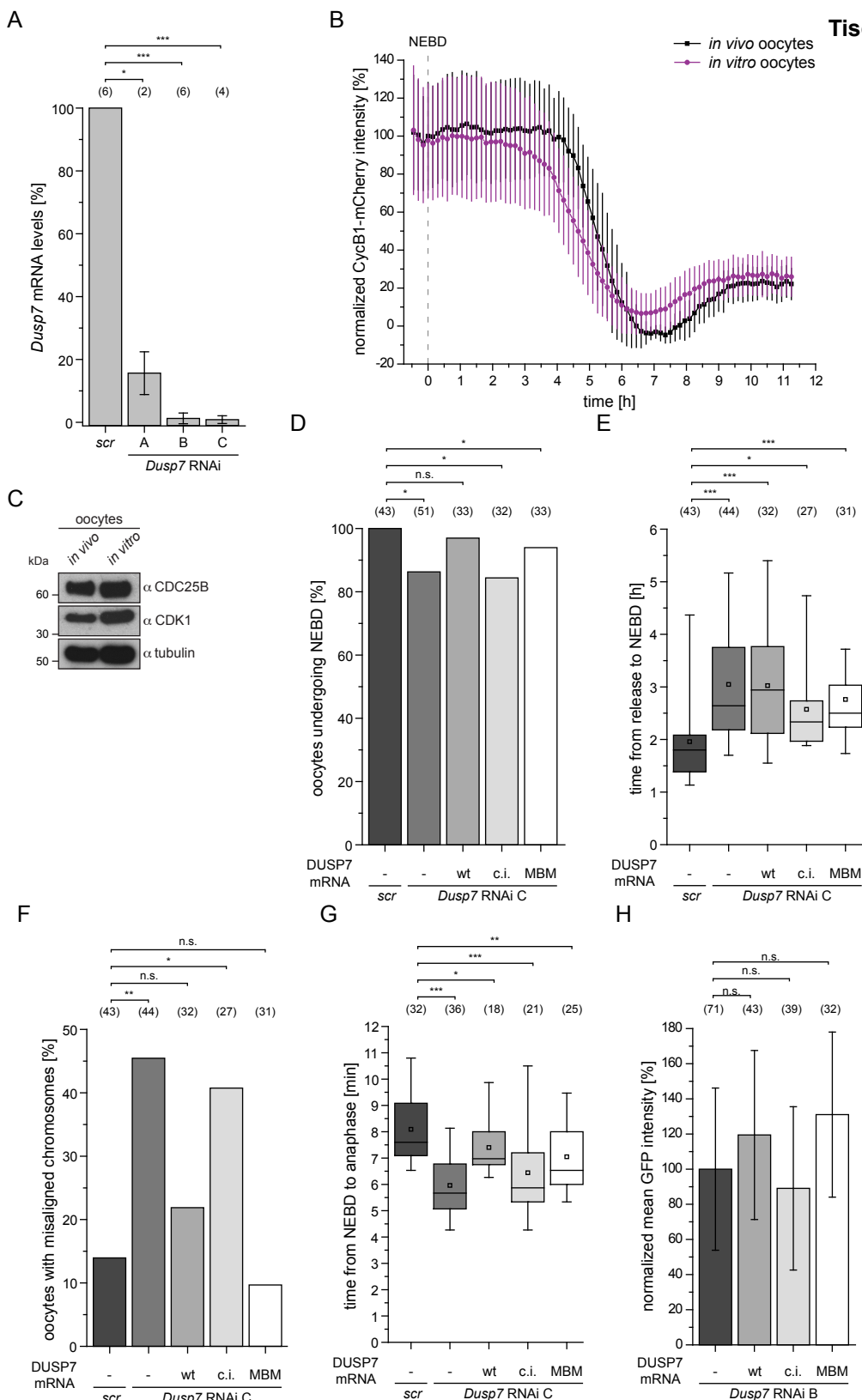
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Supplemental Figures S1 – S4 (including figure legends): All Supplemental Figures refer directly to the main Figures with the same number (e.g. Supplemental Fig. S1 is related Fig. 1).



Supplementary Figure S1 (Related to Figure 1)

(A) Quantitative real time PCR to analyze the levels of *Dusp7* mRNA after DUSP7 depletion. Due to the lack of suitable antibodies, we were unable to directly assess our depletion efficiency of DUSP7 by immunoblotting. Therefore we evaluated the depletion based on the remaining *Dusp7*-mRNA levels by quantitative real time PCR. Out of the three tested siRNA-oligos we had two that very efficiently depleted DUSP7 and showed a comparable phenotype (Fig. 1 and S1). For all further experiments we used oligo "B". To ensure specificity and reproducibility we repeated all experiments shown in Fig. 1 also with oligo "C" (see D - G). We did not observe any differences in the behavior of both siRNA oligos. The experiment was repeated three times.

(B) Follicles were isolated and cultured for 10 days *in vitro*. Oocytes from mature mice from the same strain as the follicles were isolated at the day of the experiment (*in vivo*). *In vitro* and *in vivo* grown oocytes were injected with H2B-mCherry to mark the chromosomes and Cyclin B1-mCherry. After expression, all oocytes were released from prophase arrest and imaged under a confocal microscope. The Cyclin B1-mCherry fluorescence was measured as described in (Lane and Jones, 2014). The average fluorescence intensity at NEBD was set to 100%. The CycB1 degradation dynamics of *in vivo* and *in vitro* grown oocytes are similar. The experiment was repeated three times.

(C) Immunoblot of *in vivo* and *in vitro* grown oocytes. Oocytes were isolated as described in (B) but not injected. Oocytes were lysed in laemmli sample buffer. Proteins were separated by SDS PAGE and immunoblotted for the indicated cell cycle proteins. 30 *in vivo* grown and 40 *in vitro* grown oocytes were loaded. All tested proteins are present at similar levels in *in vivo* and *in vitro* grown oocytes.

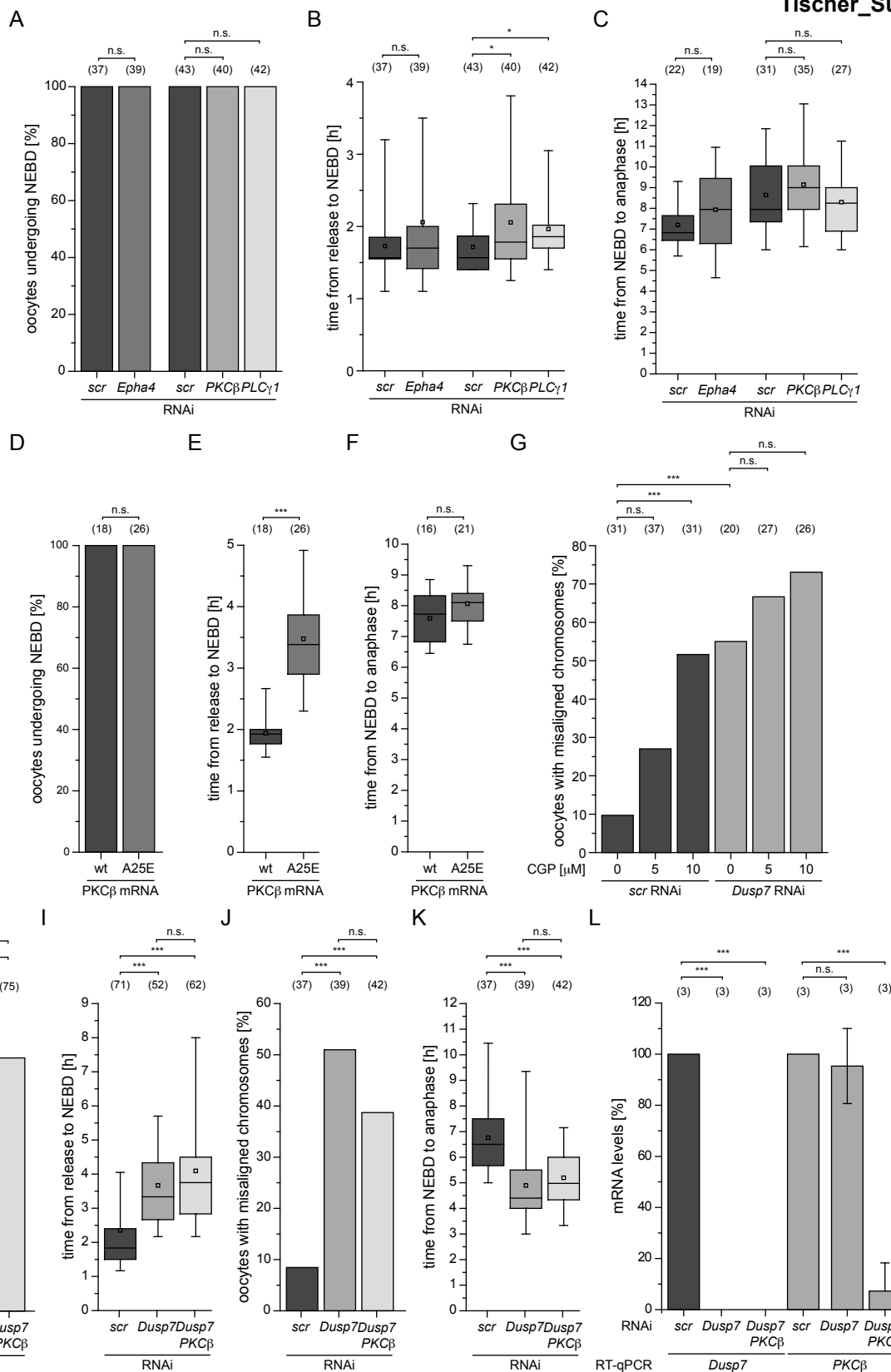
(D) Follicles were isolated and microinjected with scrambled (*scr*) or *Dusp7* RNAi oligo C. After ten days of culture the surrounding somatic cells were removed and the mature oocytes were injected again with H2B-mCherry to label the chromosomes and eGFP-MAP4 to label the meiotic spindle and analyzed for NEBD. The rescue constructs were wildtype DUSP7 (wt), catalytically inactive DUSP7 (c.i.), and MAPK binding mutant DUSP7 (MBM). GFP expression denotes expression of no DUSP7 rescue construct. The experiment was repeated five times.

(E) Oocytes from (D) were analyzed for the time from release from prophase arrest to NEBD.

(F) Oocytes from (D) that underwent NEBD were analyzed for the presence of misaligned chromosomes in the frame before the onset of anaphase.

(G) The same oocytes as in (D) were used. The time in hours that the oocytes needed from NEBD to anaphase was analyzed.

(H) The mean intensity of the GFP signal from the oocytes used in Fig. 1 B was measured. All intensities were normalized to the corresponding GFP-only expressing oocytes (-) from the same experiment. The mean from all oocytes +/- s.d. is shown.



Supplementary Figure S2 (Related to Figure 2)

(A) Follicles were isolated and microinjected with scrambled (*scr*), *Epha4*, *PKCβ*, or *PLCγ1* RNAi. After ten days of culture the surrounding somatic cells were removed and the mature oocytes were microinjected again with H2B-mCherry to label the chromosomes and eGFP-MAP4 to label the meiotic spindle. The oocytes were imaged on a confocal microscope and analyzed for NEBD at the end of the imaging. The experiment was repeated three times.

(B) Oocytes from (A) were analyzed for the time from release from prophase arrest until the oocytes underwent NEBD.

(C) The same oocytes as in (A) were used. The time that the oocytes needed from NEBD to anaphase onset was analyzed.

(D) Mature oocytes were microinjected with H2B-mCherry and GFP-*PKCβ*^{wt} or GFP-*PKCβ*^{A25E}. After mRNA expression the oocytes were released from prophase arrest, imaged on a confocal microscope and analyzed for NEBD at the end of the imaging. The experiment was repeated three times.

(E) The oocytes from (D) were analyzed for their time from release from prophase arrest until the oocytes underwent NEBD. We believe that simultaneous activation of several cPKC isoforms in PMA treated oocytes results in a much stronger defect compared to over-expression of only one active isoform.

(F) Oocytes from (D) were analyzed for the time they spend from NEBD to anaphase onset.

(G) Oocytes from Fig. 2 E were analyzed for the presence of misaligned chromosomes in the frame before anaphase onset. We were reluctant to use even higher concentrations of the inhibitor since we observed an increase in misaligned chromosomes already in control oocytes.

(H) Follicles were isolated and microinjected with scrambled (*scr*), *Dusp7*, and *Dusp7* + *PKCβ* RNAi. After the culture the mature oocytes were microinjected again with H2B-mCherry to label the chromosomes and eGFP-MAP4 to label the meiotic spindle. The oocytes were imaged on a confocal microscope and analyzed for NEBD. The experiment was repeated six times.

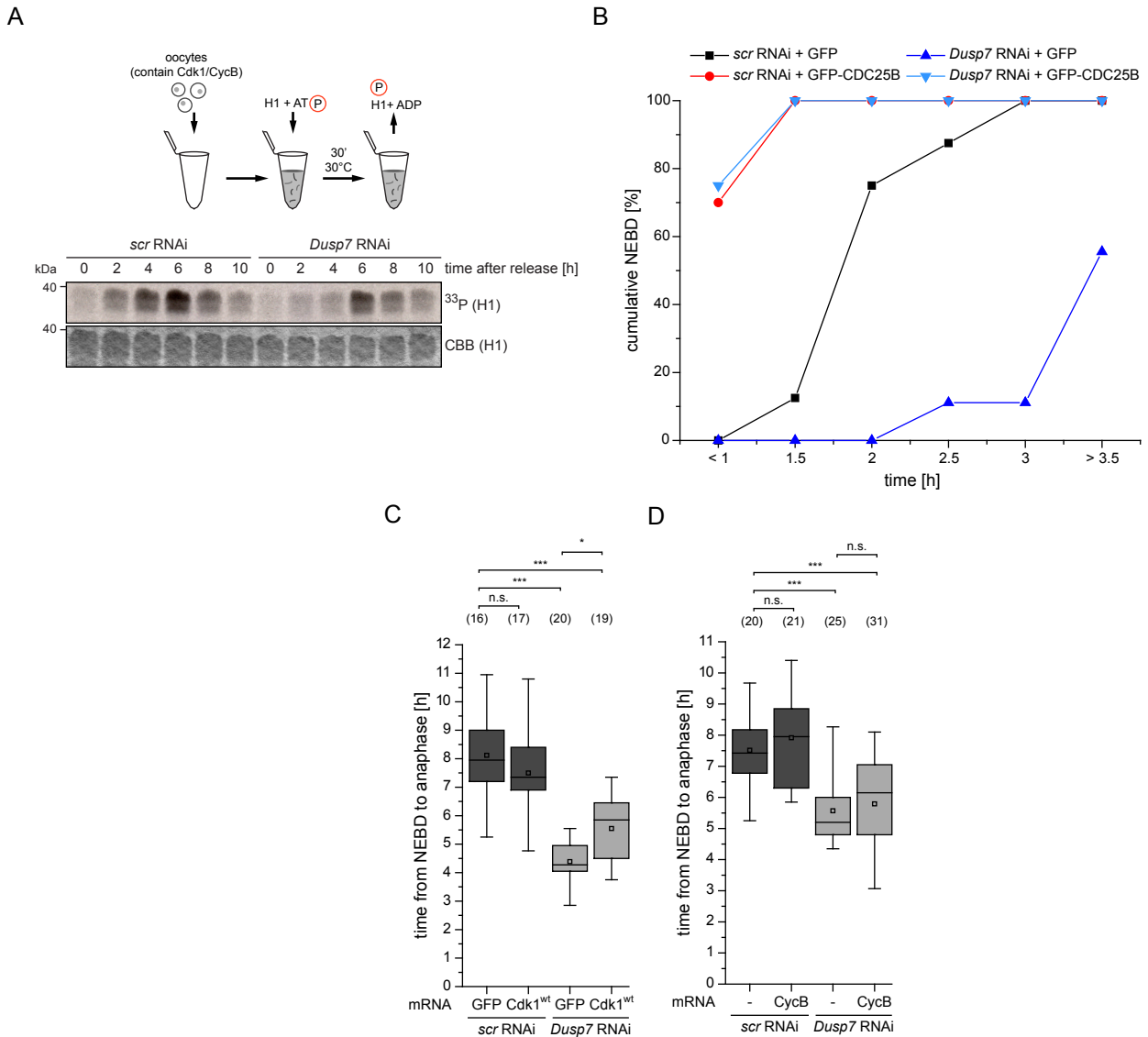
(I) Oocytes from (H) were analyzed for the time they needed from release from prophase arrest to NEBD.

(J) Oocytes from (H) that performed NEBD were analyzed for misaligned chromosomes in the frame before anaphase onset.

(K) Oocytes from (H) were analyzed for the time they spend from NEBD to anaphase onset.

(L) Quantitative real time PCR to analyze the levels of *Dusp7* and *PKCβ* mRNA after *DUSP7* depletion or co-depletion of *DUSP7* and *PKCβ*.

(M) Immunoblot showing the phospho-specificity of the pT641 *PKCβ*2 antibody. Oocytes were lysed in PMP buffer (NEB) containing 1mM MnCl₂ and complete protease inhibitors and were either treated for 1h at 30 °C with 100U lambda phosphatase (λ-PPTase) or BSA. 30 oocytes per lane were used.



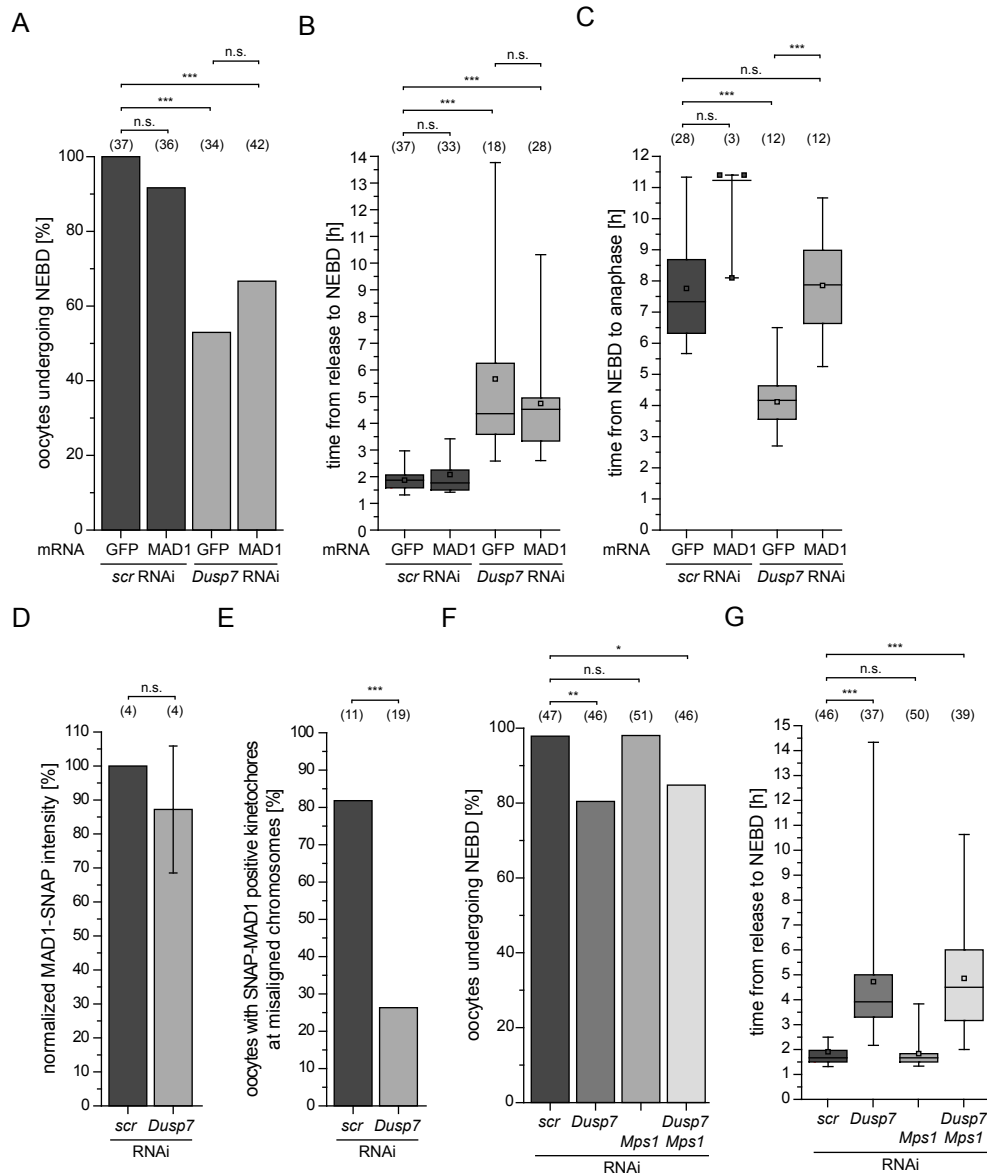
Supplementary Figure S3 (Related to Figure 3)

(A) Scheme showing the *in vitro* Cdk1/CycB kinase assay. Follicles were microinjected with scrambled (*scr*) or *Dusp7* RNAi. After 10 days of culture the oocytes were released and snap frozen at the indicated time points. The autoradiogram shows the incorporation of radioactive phosphate into Histone H1. Coomassie Brilliant Blue (CBB) staining shows the equal loading of Histone H1.

(B) Follicles were microinjected with scrambled (*scr*) or *Dusp7* RNAi. After the culture the oocytes were isolated and microinjected again with H2B-mCherry and GFP or GFP-CDC25B. Oocytes expressing CDC25B performed NEBD already before imaging at the microscope was started and analysis of the timing was therefore not possible. Instead, all oocytes that performed NEBD within a time frame of 60 minutes after release (and within 30 minute intervals afterwards) were grouped together. The cumulative percentage of oocytes that performed NEBD after the indicated time is shown. The experiment was repeated two times.

(C) Oocytes from Fig. 3 E were analyzed for the time they spend from NEBD to anaphase onset.

(D) Oocytes from Fig. 3 I were analyzed for the time they spend from NEBD to anaphase onset.



Supplementary Figure S4 (Related to Figure 4)

(A) Oocytes from Fig. 4 B were analyzed for the presence of NEBD at the end of the imaging.

(B) Oocytes from Fig. 4 B were analyzed for the time from release from prophase arrest to NEBD.

(C) Oocytes from Fig. 4 B that underwent NEBD were analyzed for the time from NEBD to anaphase onset. Note that for *scr*-RNAi + MAD1 expression only three oocytes underwent anaphase, because MAD1 over-expression arrests oocytes in metaphase. For this reason we opted to show the three single data points instead of the normal box plot.

(D) In oocytes from Fig. 4 A and B MAD1-SNAP intensity was measured in an area around the chromosomes 60 min after NEBD and corrected for background signal. To signal intensity was normalized for all individual experiments against *scrambled* (*scr*) RNAi injected oocytes. The mean normalized intensity of all experiments was calculated and is shown as mean +/- s.d.. MAD1-SNAP expression levels are not significantly different between *scrambled* (*scr*) and *Dusp7* RNAi treated oocytes. n is the number of experiments.

(E) Oocytes from Fig 4 A and B were analyzed for the presence of SNAP-MAD1 on transiently misaligned chromosomes upon formation of the metaphase plate.

(F) Oocytes from Fig. 4 C were analyzed for the presence of NEBD at the end of the imaging.

(G) Oocytes from Fig. 4 C were analyzed for the time from release from prophase arrest to NEBD.

Supplemental Table S5 (Related to Figure 2): Hits from the *in vitro* peptide screen

Microsoft Excel file containing all hits from the *in vitro* phospho-peptide screens.

Supplemental File S6 (Related to Figure 2): Input data for the Venn diagram

Input file for the website <http://www.interactivenn.net/> to re-create an interactive Venn diagram with the hits from the *in vitro* phospho-peptide screen.

Supplemental Movie S1 (Related to Figure 1): Full movie from the exemplary images in Fig. 1 A (top row). DNA is labeled with H2B-mCherry and shown in magenta. Microtubules are labeled with MAP4-eGFP and shown in green. The oocyte also expresses free eGFP, shown in green.

Supplemental Movie S2 (Related to Figure 1): Full movie from the exemplary images in Fig. 1 A (middle row). DNA is labeled with H2B-mCherry and shown in magenta. Microtubules are labeled with MAP4-eGFP and shown in green. The oocyte also expresses free eGFP, shown in green.

Supplemental Movie S3 (Related to Figure 1): Full movie from the exemplary images in Fig. 1 A (bottom row). DNA is labeled with H2B-mCherry and shown in magenta. Microtubules are labeled with MAP4-eGFP and shown in green. The oocyte also expresses free eGFP, shown in green.

Supplemental Movie S4 (Related to Figure 4): Full movie from the exemplary images in Fig. 4 A (top row). DNA is labeled with H2B-mCherry and shown in magenta. MAD1-SNAP was labeled with SNAP-Cell 505 Star (New England Biolabs) and is shown in green.

Supplemental Movie S5 (Related to Figure 4): Full movie from the exemplary images in Fig. 4 A (bottom row). DNA is labeled with H2B-mCherry and shown in magenta. MAD1-SNAP was labeled with SNAP-Cell 505 Star (New England Biolabs) and is shown in green.