**Wwc2** is a novel mitotic/meiotic cell-cycle regulator and cell fate related gene, during preimplantation mouse embryo development and oogenesis.

Giorgio Virnicchi\(^1\)*, Pablo Bora\(^1\), Lenka Gahurová\(^1\), Andrej Šušor\(^2\) and Alexander W. Bruce\(^1\)*

\(^1\)Laboratory of Early Mammalian Developmental Biology (LEMDB), Department of Molecular Biology & Genetics, Faculty of Science, University of South Bohemia, Branišovská 31, 370 05 České Budějovice (Budweis), CZECH REPUBLIC.

\(^2\)Laboratory of Biochemistry and Molecular Biology of Germ Cells, Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Rumburská 89, 277 21 Liběchov, CZECH REPUBLIC.

*correspondence; A.W.B. awbruce@prf.jcu.cz & G.V. giorgio.virnicchi@gmail.com

Tel: +420387772291
Fax: +42038777226 (not confidential)

Keywords: Meiosis, mitosis, chromosome segregation, cytokinesis, cell division, cell fate, preimplantation mouse embryo, blastocyst, oocyte.
ABSTRACT

Derivation of late stage mouse blastocysts (E4.5) marks the end of the preimplantation period, whereby the earliest developmental events are manifest in regulated cell cleavage divisions and formation of three distinct cell lineages. Here we report targeted dysregulated expression of the Wwc2 gene, an ill characterised paralog of the upstream Hippo-signalling activator Kibra/Wwc1, is specifically associated with cell autonomous deficits in embryo cell number and gross division abnormalities/phenotypes; typified by imbalanced daughter cell chromatin segregation. Clonal dysregulation additionally implicates Wwc2 in maintaining the pluripotent epiblast lineage by the late blastocyst stage. The uncovered early mitotic regulatory role is conserved during mouse oocyte meiotic maturation, whereby Wwc2 dysregulation blocks progress to the fertilisation competent stage of meiosis II metaphase arrest, with attendant spindle defects and failed Aurora kinase A (AURKA) phosphorylation/activation. All identified Wwc2 specific cell division phenotypes are fully revertible by expression of recombinant HA-epitope tagged WWC2, that localises to cytokinesis derived mid-body structures and restores activated p-AURKA levels, in embryos and oocytes, respectively. Thus, we have identified Wwc2 as a novel regulator of both meiotic and early mitotic cell divisions, and subsequently mouse blastocyst cell fate.
INTRODUCTION

Mouse embryo development begins with the fertilisation of ovulated secondary oocytes, appropriately arrested in the metaphase of the second meiotic division (MII-arrested), resulting in the diploid and developmental competent zygote (1). Ovulated secondary MII oocytes arise from subpopulations of primary oocytes, that have been arrested in the dictyate stage of prophase of meiosis I (MI) since prenatal development, stimulated by maternal reproductive hormones to re-enter and complete meiosis I (reviewed 2). In addition to ensuring both the necessary growth required to support later embryonic development and expression of gene products required to execute meiosis, meiotic maturation ensures replicated chromosomal bivalents are appropriately resolved and faithfully segregated between the first polar body and a developmentally competent secondary MII oocyte (endowed with the capacity to subsequently faithfully segregate sister chromatids between the second polar body and resulting zygote, post-fertilisation). Failure to precisely segregate chromosomes, resulting in egg and/or zygotic aneuploidy, has severe and usually terminal consequences for embryonic development, with aneuploidy attributable to the human female germline recorded as the leading single cause of spontaneously aborted pregnancy 3, 4. An extensive literature covering the varied and integrated molecular mechanisms that underpin the germane segregation of homologous chromosomes in MI exists; ranging across meiotic cell-cycle resumption, germinal vesicle (GV/nuclear envelope) breakdown, meiotic spindle assembly, spindle microtubule-kinetochore attachment, chromosomal congression, functioning of the spindle assembly checkpoint (SAC), regulation of the anaphase promoting complex/cyclosome (APC/C) and regulation of cytokinesis/polar body generation (see comprehensive reviews 2, 5, 6, 7, 8). In keeping with all mammals, and unlike most mitotic somatic cells, meiotic spindle formation in primary mouse oocytes occurs in the absence of centrioles/centrosomes. Spindle assembly is initiated in the vicinity of condensing chromosomes from coalescing microtubule organising centres (MTOCs) and is further stabilised by chromosome derived RAN-GTP gradients that respectively promote and inhibit microtubule polymerisation and destabilisation 5, 6, 8, 9, 10. Unlike other mammalian developmental systems, the transition from MTOC initiated spindle formation to centrosomal control in mice only occurs by the mid-blastocyst stage, when centrosomes appear de novo (11); contrasting with other mammals (e.g. humans) were fertilising sperm provide a founder centrosome that ensures the first mitotic spindle is assembled centrosomally 12, 13. Thus, formation and functioning of the first meiotic, and somewhat uniquely, early mitotic spindles in mice are subject to non-centrosomal based regulation. Amongst known key regulators of spindle dynamics are the conserved Aurora-kinase family proteins (AURKA, AURKB & AURKC, collectively referred to here as AURKs) that all exhibit germ cell and early embryonic expression; although, AURKC is not expressed in other somatic cells 14. During meiosis, AURKs impart important regulatory roles in spindle formation/organisation, MTOC clustering, chromosome condensation and alignment, plus correct microtubule-kinetochore attachment, chromosomal cohesion and cytokinesis in mitosis (reviewed in 15). Specifically, AURKA protein is essential for MI progression 16 and is continually localised with MTOCs and MI/ MII spindle poles, regulating clustering and initiating microtubule nucleation/dynamics, throughout meiosis 15, 17, 18, 19; with similar roles in post-fertilisation zygotes 20. Despite the lack of centrosomes, AURKA functionally cooperates with the key centrosomal protein Polo-like kinase 4 (PLK4), to promote meiotic spindle microtubule nucleation 21 and PLK4 participates in MTOC mediated/non-centrosomal mitotic divisions of cleavage stage mouse embryo blastomeres 22.
Over the 4.5 days post MII oocyte fertilisation, a series of asynchronous cell cleavage divisions generates blastocysts capable of uterine implantation, comprising three distinct cell lineages; an outer-residing, differentiating and apical-basolaterally polarised epithelium called the trophectoderm (TE – a progenitor of placental tissue), differentiating primitive endoderm (PrE – precursors of the yolk sac), represented as a polarised superficial monolayer of inner-cell mass (ICM) cells in contact with the fluid filled cavity and the pluripotent epiblast (EPI – progenitors of subsequent foetal tissue), found completely encapsulated within the ICM (comprehensively reviewed 23, 24, 25, 26, 27, 28). Characteristically, the cell-cycle dynamics of mouse (indeed, mammalian) preimplantation development are generally distinct from other metazoan species, being atypically long by comparison (reviewed in 29), comprising 12-14 hours from the 4-cell (E2.0) stage 30, 31, 32, 33, versus the much more rapid early embryonic divisions observed of oviparous species 34, 35, 36. Elongated early cell-cycles are necessary to facilitate mechanisms of selective embryonic differentiation (towards supportive extraembryonic tissues) required to sustain viviparous development 37. Amongst such mechanisms is the differential regulation of Hippo-signalling, (originally identified in Drosophila as a cell proliferation and tissue growth/size regulating pathway but now implicated in varied developmental/pathological paradigms 38), which has emerged as an important means of correctly specifying the emerging blastocyst lineages. Specifically, that encapsulated and apolar inner-cells of the nascent ICM, generated after the 8- to 16- and 16- to 32-cell stage transitions, form active Hippo-signalling centres at adherens junctions (AJs) that potentiate the terminal core pathway kinases, LATS1/2 (in a mechanism requiring recruitment of the essential activator AMOT 39, 40, 41) to phosphorylate the transcriptional cofactors effectors YAP and WWTR1/TAZ (collectively referred to here as YAP). Combined YAP phosphorylation (promoting cytoplasmic 14-3-3- proteins binding) and AMOT interaction prevents YAP nuclear accumulation that ensures members of the TEA-domain containing transcription factors (specifically TEAD4 in this context) are unable to activate TE-specific genes (e.g. Cdx2 or Gata3) 40. Conversely, AJ-localised Hippo-pathway activation at basolateral membranes of polarised outer-cells is actively suppressed via AMOT protein sequestration to the cell contactless apical pole permitting unphosphorylated YAP to enter the nucleus to molecularly endow TEAD4 to appropriately transcriptional activate TE-related gene expression. Therefore, the initial inheritance, or otherwise, of an apical pole enriched in apical polarity factors (e.g. aPKC-Par complex) defines whether dividing 8-cell (E2.5) or outer 16-cell (E3.0) stage progeny blastomeres, respectively suppress or activate Hippo-signalling and hence exhibit pluripotency (as an inner-cell) or initiate TE differentiation (as an outer-cell). Indeed, experimental strategies to disrupt outer-cell apical-basolateral polarity 42, 43, 44, 45, or abrogate Tead4 expression 46, 47, 48, result in failed outer-cells TE specification and ectopic ICM pluripotency-related marker gene expression (reviewed 49, 50). Interestingly, YAP enabled TEAD4 complexes simultaneously suppress pluripotent gene expression (e.g. Sox2) in derived outer-cells 51, 52 and prevent precocious Sox2 expression prior to the 16-cell stage 53. Additionally, experimentally activated Hippo-signalling (i.e. LATS1/2 over-expression, to antagonise YAP-TEAD4 formation) can potentiate Sox2 expression (even in outer-cells) by the 32-cell/ early blastocyst stage 51. However, eventual EPI specification by the late blastocyst stage, actually requires later YAP translocation into the nucleus, in a process that is inherently heterogeneous and leads to competitive apoptotic elimination of EPI progenitors exhibiting reduced naïve pluripotency-related gene expression (and low nuclear YAP) 54. Collectively, these data illustrate the integral role of Hippo-signalling in regulating key cell fate events in early mouse embryogenesis and suggest potential functional roles for other, potentially novel, factors capable of interacting with core Hippo-pathway components.
The WW-domain and C2-domain containing (WWC-domain) protein KIBRA was originally identified as an interactor of Dendrin, a modulator of post-synaptic cytoskeleton. Subsequently, Kibra was reported as a positive regulator of Hippo-signalling in *Drosophila* tumour suppressor/cell proliferation screens by phosphorylating the fly orthologue of mammalian LATS1/2, *warts/Wts*; subsequently confirmed by KIBRA-LATS1/2 interactions in mammalian cells. In contrast to *Drosophila*, tetrapod genomes typically encode three WWC-domain paralogs (*KIBRA*/WWC1, WWC2 and WWC3; *Mus musculus* genome only contains *Wwc1/Kibra*, hereon referred to as *Kibra*, and *Wwc2* owing to evolutionarily recent chromosomal deletion of the ancestral *Wwc3*). Human KIBRA shares 49% and 40% amino acid identity WWC2 and WWC3, respectively, and all paralogs are cable of homo- and heterodimerisation (in all combinations). All three paralogs can also activate Hippo-signalling (causing LATS1/2 and YAP phosphorylation) in inducible cell line models and cause the Hippo-related *Drosophila* ‘rough-eye’ phenotype, caused by reduced cell proliferation, when over-expressed in the developing fly eye. The WW-domain (found as N-terminal tandem repeats in KIBRA and WWC2/3) is one of the smallest known protein domains, comprising 35 amino acids and interacts with short proline rich motifs (~4-6 amino acids) containing the consensus sequence, L/PPxY. Interestingly, WW-domains and L/PPxY-motifs are enriched in confirmed Hippo-signalling-related proteins and in potential regulator/effectors identified in comprehensive proteomic assays of the human Hippo-pathway interactome. Despite a comparatively large pan-model KIBRA-related Hippo-pathway activator literature, the roles of WWC2/3 are understudied and restricted to limited reports consistent of tumour suppressor function in specific cancers (e.g. hepatocellular carcinoma and epithelial-mesenchymal lung cancers). Therefore, due to the importance of differential/regulated Hippo-signalling in early mouse development and the collective paucity of WWC-domain gene related data within this developmental window, we investigated the effect of dysregulating *Kibra* and *Wwc2* expression in mouse preimplantation embryos.

We report specific disruption of *Wwc2* expression in mouse embryo blastomeres results in cell autonomous division and cleavage phenotypes associated with mis-segregated chromosomes and defective cytokinesis (not replicated by targeted dysregulation of *Kibra*). The collective defects yield blastocysts with fewer overall cells, increased ICM apoptosis and reduced capacity to populate pluripotent EPI; indicating a potential pluripotency regulating role. Observed phenotypes were rescued by the expression of epitope-tagged recombinant WWC2 protein, that localised within the persistent mid-body structures between blastomeres post cleavage/division. Furthermore, we report similarly rescuable cell division phenotypes associated with impaired *Wwc2* expression in meiotically maturing mouse primary oocytes, that are coincident with profound spindle defects and a block in activating/phosphorylating the key cell-cycle regulatory kinase Aurora-A (AURKA). Accordingly, we conclude *Wwc2* is a novel regulator of both female acentrosomal meiotic and early embryonic mitotic cell division and mouse blastocyst cell fate.
RESULTS

WWC-domain containing genes are expressed in preimplantation mouse embryos

As introduced above, in the context of the central role of Hippo-signalling in mouse preimplantation embryo development \(^{49}\), we investigated the potential regulatory role of WWC-domain containing genes (i.e. Kibra and Wwc2 in mice \(^{60}\)) in blastocyst formation. Accordingly, we first assayed Kibra and Wwc2 mRNA expression in preimplantation mouse embryos microinjected with non-specific control dsRNA or constructs specific for each gene (Fig. 1a). Stable levels of Kibra and Wwc2 transcripts were readily detectable at both 8- (E2.5) and 32-cell (E3.5) stages, with normalised Wwc2 expression twice as abundant as Kibra. Each transcript level could be robustly reduced using dsRNA mediated global knockdown at both assayed stages (note, a lack of available antibodies prevented protein expression assays). We next assayed for overt developmental defects associated with individual or combined Kibra/Wwc2 knockdown by microinjecting dsRNA(s) in one blastomere of 2-cell (E1.5) stage embryos, culturing to the late blastocyst (E4.5) stage and counting total cell number (Fig. 1b). Kibra gene knockdown had no significant effect on total cell number, or embryo morphology (versus control dsRNA), but a severe attenuation in cell number when targeting Kibra and Wwc2 transcripts in combination was observed. Interestingly, this defect was statistically indistinguishable from the group of embryos microinjected with Wwc2 dsRNA alone, indicating sole knockdown of the Wwc2 paralog was sufficient to induce the observed phenotype (note, Kibra mRNA levels were unaffected by Wwc2 dsRNA by the 8-cell/E2.5 stage, whereas Wwc2 transcripts were robustly reduced, confirming Wwc2 dsRNA specificity – Fig. 1a and supplementary Fig. S1). Repetition of the Wwc2 knockdown, in a fluorescently marked clone (whereby rhodamine conjugated dextran beads/ RDBs were co-injected with Wwc2 siRNA) confirmed reduced cell numbers as early as the 16-cell (E3.0) stage, specifically within the marked clone that itself exhibited reduced contribution to the initial inner-cell/ ICM founding population (Fig. 1c). Accordingly, we decided focus our further investigations on the overt phenotype associated with Wwc2 knockdown.

Embryo cell number deficits caused by Wwc2 knockdown are associated with defective cell division.

Although the utilised Wwc2 dsRNA was carefully designed to avoid off target effects (with specific respect to Kibra expression, Figs. 1a & S1), we sought to phenocopy our observations using an siRNA mediated approach (targeting an alternative region of the Wwc2 mRNA – Fig. S1); thus, providing added confidence to our initial observations and affording the option of phenotypic rescue, by expressing recombinant siRNA-resistant Wwc2 mRNA. Figure 2 confirms the selected Wwc2 siRNA near completely eliminated detectable Wwc2 transcripts, after global knockdown, in 32-cell (E3.5) stage blastocysts. Moreover, subsequent to the microinjected 2-cell (E1.5) stage, an assay of total cell number at all cleavage stages up to the late blastocyst (E4.5) stage revealed an accumulative and statistically robust deficit beginning after the 8-cell (E2.5) stage (Fig. 2c). Indeed, from the equivalent 8-cell (E2.5) to 32-cell (E3.5) stages (judged by control embryo development), total cell number did not significantly increase in Wwc2 knockdown embryos but did start to increase during the blastocyst maturation period (32- to >64-cell, as judged in control embryo groups; i.e. E3.5 – E4.5 stages). Significantly, it was not unusual for the Wwc2 knockdown embryos to initiate cavitation (not shown). We also assayed if the Wwc2 siRNA phenotype was cell autonomous by creating RDB marked Wwc2 specific siRNA knockdown clones (comprising 50% of the embryo – Fig. S2). As expected, we observed a statistically significant Wwc2 siRNA mediated deficit in cell number within the
microinjected clone (versus the non-microinjected sister clones and the equivalent microinjected clone in control siRNA embryos) at all stages, from the 8-cell (E2.5) to the late blastocyst (E4.5). However, we did not observe significant differences between control and \textit{Wwc2} knockdown groups in the number of cells within the non-microinjected clone (with the exception of the 32-cell (E3.5) stage where there were an average of 2.3 fewer cells in the \textit{Wwc2} knockdown group). These data support a novel and cell autonomous role for \textit{Wwc2} in regulating appropriate cell number during preimplantation mouse embryo development. Whilst assaying cell numbers, we observed a number of nuclear/chromatin morphological abnormalities within \textit{Wwc2} knockdown embryos, not evident in control embryos. Illustrative examples, at the equivalent of the 32-cell (E3.5) stage are provided (Fig. 3b) and were categorised as; ‘abnormal nuclear morphology’ (including that typical of persistent ‘association with the mid-body’), associated with ‘cytokinesis defects’ (typified by bi-nucleated cells) or coincident with ‘multiple or micronuclei’.

Accordingly, we calculated the frequencies by which each, or a composite of, the stated abnormalities were observed (compared to control siRNA conditions) at each assayed cleavage stage; defined per embryo (in at least one blastomere) or per individual assayed cell (Fig. 3c). Except from a single incidence of an early blastocyst stage cell exhibiting a single micronucleus, no other abnormalities were observed in any control siRNA microinjected embryo, at any developmental stage. Conversely, abnormally shaped nuclei were present in over a quarter of \textit{Wwc2} knockdown embryos at the 8-cell (E2.5) stage and were found in all assayed embryos by the equivalent late blastocyst (E4.5) stage. A similar trend, from the 16-cell (E3.0) stage, was observed in relation to multiple/micronuclei. Despite being less prevalent, cytokinetic defects affected nearly a quarter of \textit{Wwc2} knockdown embryos by the late blastocyst (E4.5) stage. Indeed, all \textit{Wwc2} knockdown embryos exhibited one or more defect, in at least one cell, by the mid-blastocyst (E4.0) stage, with the collective defects (excluding cytokinesis/bi-nucleation) first arising in just over a quarter of 8-cell stage (E2.5) embryos. When we analysed the same phenotypes on the level of each individually assayed blastomere, we found that just over a quarter of cells were affected by the late blastocyst (E4.5) stage; however, it should be noted that such embryos comprised a much smaller average of overall cells, versus control siRNA groups \textit{i.e.} 31.3±3.0 against 87.0±3.8 - Fig. 2c). Hence, our collective interpretation is \textit{Wwc2} knockdown in cleavage stage mouse embryos is associated with cell autonomous division defects that contribute to embryos with progressively fewer constituent blastomeres as preimplantation development proceeds past the 8-cell stage; invoking a role for \textit{Wwc2} in regulating appropriate cell division/mitosis in blastomeres of the early mouse embryo.

\textbf{siRNA mediated \textit{Wwc2} knockdown affects blastocyst cell fate derivation.}

As described, cell number deficit/defective cell division phenotypes were first evident in global siRNA mediated \textit{Wwc2} knockdown embryos around the 8- to 16-cell stage (Figs. 2 & 3). Moreover, clonal dsRNA mediated \textit{Wwc2} knockdown analysis was specifically associated with reduced inner-cell clone numbers (Fig. 1c). As the transition from the 8- to 16-cell stage, represents the first developmental point constituent embryonic cells are overtly distinct, both in terms of intra-cellular apical-basolateral polarity and relative spatial allocation, with consequences for ultimate blastocyst cell fate \textit{i.e.} comprising polarised outer-cells that can give rise to both TE and further inner-cells, plus apolar inner ICM progenitors 66), we assayed if clonal \textit{Wwc2} knockdown altered TE versus ICM cell fate by the early blastocyst (E3.5) stage. The need to assay the first cell fate decision, in this context, was further reinforced by cited precedents implicating \textit{Kibra}, and hence by association its paralog \textit{Wwc2}, in activating Hippo-signalling in flies and mammals 56, 57, 58, 59, 60, itself central to TE /ICM specification in mouse preimplantation embryos 49.
Accordingly, we created stably marked clones of $W_{\text{wc2}}$ knockdown and control cells (by expressing recombinant GAP43-GFP as an injection membrane marker) and assayed expression of the Hippo-sensitive TE marker protein CDX2 (Fig. 4). Control siRNA treated embryos developed appropriately to yield 32-cell (E3.5) stage blastocysts consisting an average of 59% outer (CDX2 positive) and 41% inner (CDX2 negative) cell populations, with a statistically equal contribution from each clone (i.e. either control siRNA microinjected or non-microinjected derived). As expected, $W_{\text{wc2}}$ siRNA microinjected embryos comprised significantly fewer cells with the microinjected clone also characterised by the previously observed abnormal nuclear morphologies and a robustly significant impaired contribution to the ICM; on average 1.6±0.2 cells versus 6.8±0.3 in the non-microinjected clone (or 7.3±0.4 or 7.4±0.3 in the respective clones of control siRNA microinjected embryos). Clonal TE contribution was also significantly impaired but to a much lesser degree and the potential TE deficit was compensated by an increased contribution from the corresponding non-microinjected clone. We also observed a small outer-cell population of $W_{\text{wc2}}$ knockdown clones that failed to express CDX2 (~20%; not observed in control embryos) with the remaining outer-cells often exhibiting comparatively reduced CDX2 immunoreactivity, when compared to both the non-microinjected clone or either clone in the control siRNA microinjected groups (concurrently immuno-stained and imaged using identical protocols; Fig. 4b). However, no ectopic CDX2 expression within inner-cells of the $W_{\text{wc2}}$ knockdown clone was observed and the non-microinjected clone appropriately only expressed CDX2 in outer-cells.

Therefore, consequent to clonal $W_{\text{wc2}}$ knockdown, the overall percentage make up of outer and inner-cells was skewed in favour of outer TE (largely CDX2 positive – 72%) over ICM (exclusively CDX2 negative - 28%). These results suggest that the required outer-cell Hippo-pathway suppression (to specify TE) and inner-cell activity (to prevent TE differentiation and promote pluripotency) are predominantly intact within the $W_{\text{wc2}}$ knockdown clone, although maintenance of TE specification/differentiation in outer-cells is modestly impaired. Therefore, endogenous $W_{\text{wc2}}$ is unlikely to function as a centrally critical Hippo-signalling pathway (i.e. LATS1/2) regulator (as implied by previous Kibra/KIBRA related reports demonstrating a role in Warts/LATS1/2 phosphorylation/activation in Drosophila and mammalian cells [56, 57, 58, 59, 60]), in the specific context of TE versus ICM cell fate specification/segregation, prior to blastocyst formation. Indeed, such conclusions are supported by a lack of overt apical polarity (PARD6B) defects or ectopic nuclear exclusion of YAP (as the transcriptional co-activator and Hippo-pathway effector protein [40]) in outer-residing $W_{\text{wc2}}$ knockdown blastomeres, by the 32-cell (E3.5) stage (Fig. S3).

However, normal YAP nuclear exclusion in inner-residing $W_{\text{wc2}}$ knockdown cells was found to be consistently less robust than that observed in equivalent inner-cells of control siRNA microinjected embryos, possibly suggesting moderately impaired Hippo-signalling activation/cytoplasmic YAP sequestration caused by $W_{\text{wc2}}$ knockdown (Fig. S3); although the observed nuclear YAP levels were far from equivalent to those observed in outer residing blastomeres of the same embryos (Fig. S3). Such data are indicative of a potentially mild inhibition of active Hippo-signalling in such inner $W_{\text{wc2}}$ knockdown cells, by the 32-cell (E3.5) stage.

We next extended the analyses to the late blastocyst (E4.5) stage cell lineages, assaying TE and EPI markers (i.e. CDX2 & NANOG) or PrE and EPI markers (i.e. GATA4 & NANOG or GATA4 & SOX2) as pairwise combinations (Figs. 5 & S4). We again observed $W_{\text{wc2}}$ knockdown clone specific cell number deficits (relayed into reduced total blastocyst cell number) and nuclear morphology/cell division related phenotypes. Despite such defects, and contrasting with the early blastocyst stage, the ratio of total outer (TE) to inner (ICM) cells was not significantly different between $W_{\text{wc2}}$ or control siRNA microinjection groups (across all assayed lineage combinations; Fig. 5d-
f); indicating a degree of regulative development during blastocyst maturation. However, collectively we noted incidences of fragmented nuclei, indicative of apoptotic cell death, that were significantly more prevalent within the ICM of Wwc2 siRNA, versus control siRNA, microinjected embryo groups. Moreover, the observed apoptosis was significantly enriched within the microinjected clone of Wwc2 knockdown embryos, suggesting such ICM residing cell clones are prone to an increased probability of cell death (Fig. 5b). Consistently, the overall and significantly reduced contributions of Wwc2 siRNA derived clones were more pronounced for ICM versus outer-cell populations (Fig. S4). Focussing, on CDX2 and NANOG immuno-stained groups, we again noted a population of CDX2 negative outer-cells and generally reduced CDX2 expression within the Wwc2 knockdown clone (Fig. 5c). Moreover, the significantly reduced number of ICM cells derived from the Wwc2 knockdown clone did not segregate between NANOG positive (indicative of EPI) and NANOG negative (potentially PrE) cells, as per marked control siRNA clones (i.e. according to a clone’s overall percentage contribution within the ICM), but were significantly biased to populate the potential, NANOG negative, PrE (Fig. 5d). Considering the embryos in the whole, the overall reduction in size of the potential PrE (NANOG negative) population that was associated with clonal Wwc2 knockdown, was much less than that observed in the EPI (Fig. S4). This suggests inner Wwc2 siRNA microinjection derived clones are impaired in their contribution to sustain EPI numbers, by the late blastocyst (E4.5) stage, but are able to differentiate to form PrE. This interpretation was supported by data directly assaying the two ICM lineages (i.e. GATA4 in combination with either NANOG or SOX2; Figs. 5e & f, plus Fig. S4), whereby the characteristically low number of inner Wwc2 knockdown cells/ clones observed, similarly segregated in a manner favouring the PrE (marked by GATA4) over EPI (marked by either NANOG or SOX2); note, compensatory increases in EPI contribution were also observed in the non-microinjected clone (Fig. S4). We also observed numerous examples of fragmented/ apoptotic inner-cell nuclei displaying distinct immuno-reactivity for SOX2 (Fig.5c), suggesting the increased incidence of apoptosis observed within the ICM residing and microinjected cell clones of Wwc2 knockdown embryos (Fig. 5b) is centred on specified EPI that is ultimately unable to be maintained (although it was not possible to reliably quantify the number of SOX2 positive apoptotic nuclei). Interestingly, in Wwc2 siRNA microinjected embryo groups, we also observed a small population of marked ICM clones that expressed neither PrE or EPI markers (comprising 4.6% of all ICM cells and 18.7% of the clone) that were not present in control siRNA treated groups, potentially indicative of a further impairment in ICM cell fate derivation. In conclusion, our late blastocyst cell fate analyses confirm Wwc2 knockdown clone specific and autonomous reductions in overall cell number, that more robustly affect the ICM versus TE. Such reductions are compensated for by regulation within the non-injected clone, that ultimately preserves the overall TE:ICM ratio (albeit with fewer overall cells). However, the lineage contribution of Wwc2 knockdown clones within the ICM is biased against the pluripotent EPI in favour of PrE differentiation, via an implicated mechanism possibly involving clone specific and selective EPI apoptosis; suggesting a role for Wwc2 in contributing to specified EPI maintenance that is additional to that related to cell division, yet inherently difficult to experimentally resolve.

siRNA resistant HA-Wwc2 mRNA rescues Wwc2 siRNA knockdown phenotypes; recombinant HA-WWC2 protein associates with cell division generated mid-bodies.

Despite obtaining consistent RNAi phenotypes using distinct dsRNA/siRNA constructs and their careful design aimed to avoid potential off-target effects or cross reactivity with paralogous Kibra mRNA (Figs. 1 and S1),
we sought to further verify the identified novel role of Wwc2. Accordingly, we derived a recombinant and N-terminally HA-epitope tagged Wwc2 mRNA construct (HA-Wwc2) that had been specifically mutated within the siRNA complementary sequence (yet preserving a redundant sequence of amino acid codons; see Fig. S5) to block recognition and hence remain available for translation in Wwc2 siRNA containing cells. Thus, we repeated our marked clonal microinjection experiments but added a third condition in which Wwc2 siRNA was co-microinjected with HA-Wwc2 mRNA. Microinjected 2-cell (E1.5) stage embryos were cultured until the early (E3.5) and late (E4.5) blastocyst stages and total/clonal cell contributions to inner/outer-cell populations, plus the incidence of abnormal nuclear morphology, calculated (Fig. 6). Compared with the control siRNA group, embryos microinjected with Wwc2 siRNA alone exhibited the typical clone autonomous phenotypes described above. However, under Wwc2 siRNA conditions supplemented with co-microinjected HA-Wwc2 mRNA (the expression of which was confirmed, see below and Fig. 6d) the average number and clonal/spatial composition of constituent early (E3.5) and late (E4.5) blastocyst cells was statistically indistinguishable from control siRNA microinjections groups (with the only exception being a small reduction in the ICM contribution of the non-microinjected clone by the late/E4.5 blastocyst stage; i.e. from 9.5±0.4 to 8.2±0.3). Additionally, there was a complete rescue of the incidence of nuclei with abnormal morphology (with only one exception in all blastomeres assayed – shown in Fig. 6d). Collectively, these data demonstrate the specificity of the RNAi reagents and confirm Wwc2 as a novel regulatory gene of cell division dynamics, with consequences for cell lineage derivation in preimplantation stage mouse embryo development. As referenced above, the HA-epitope tag incorporated within the confirmed HA-Wwc2 ‘phenotypic rescue’ mRNA construct not only enabled us to verify its translation but also its sub-cellular localisation within the microinjected clone, using an immuno-staining approach. Interestingly, given the clone autonomous cell number/division defects observed after Wwc2 knockdown, we consistently detected anti-HA immuno-reactivity at structures typical of mitotic spindle derived mid-bodies (generated after cytokinesis), that were particularly evident within the HA-Wwc2 microinjected clones at the 32-cell stage (Fig. 6d & Fig. S6a). As a recent study identified a critical role for uncharacteristically persistent interphase mid-bodies (referred to as ‘interphase microtubule bridges’) as important MTOCs within the blastomeres of cleavage stage embryos 65, we assayed their number (immuno-staining for α-Tubulin and activated phospho-Aurora-B/pAURKB, as recognised midbody markers – 66) in control and Wwc2 knockdown embryos at the same 32-cell stage, but could not detect any statistically significant variation in their overall incidence (when corrected for the reduced cell number caused by Wwc2 knockdown - Fig. S6c-e). Hence whilst HA-Wwc2 derived recombinant protein is associated with cell division mid-bodies, the removal of endogenous Wwc2 mRNA does not appear to impair mid-body formation or persistence following compromised cell division.

**Wwc2 mRNA depletion in primary (GV) oocytes impairs meiotic maturation.**

We next asked the question whether a similar knockdown of Wwc2 expression in meiosis I arrested germinal vesicle (GV) stage primary mouse oocytes would elicit any defects during maturation to the metaphase arrested stage of meiosis II (MII), particularly because both paradigms of cell division occur in the absence of centrioles 5, 6, 8, 9, 10, 11. We observed abundant Wwc2 transcripts in GV and MII oocytes (plus zygotes) that show evidence of becoming cytoplasmically poly-adenylated, and hence more likely to be translated as required functional proteins 69, between the GV and MII stages (as revealed using alternative, oligo-dT versus random hexamer, cDNA synthesis priming strategies - Fig. 7a); indeed consultation of our previously published assay of meiotically maturing oocyte polysome
associated transcripts\textsuperscript{70} supports this interpretation (\textapprox 40\% polysome association; Fig. 7b). We therefore confirmed our \textit{Wwc2} siRNA construct could elicit robust knockdown in microinjected GV oocytes, that had been blocked from re-entering meiosis I (using a cAMP/cGMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine/ IMBX) and then permitted them to \textit{in vitro} mature (IVM) to the MII equivalent stage (Fig. 7c & d). Such oocytes were fixed and immuno-fluorescently stained for $\alpha$-Tubulin (plus DAPI DNA stain), to assay for potential phenotypes. In our two control conditions (\textit{i.e.} non-microinjected or control siRNA microinjected oocytes) $>$90\% of GV oocytes matured to the MII arrested stage, typified by an extruded polar body (PB1) and metaphase II arrested spindle. However, in the \textit{Wwc2} knockdown group the successful IVM rate was reduced to 8.6\% and oocytes presented with various arrested phenotypes (lacking PB1) that we categorised by the presence of metaphase I meiotic spindles (-PB1 +MI spindle; 42.9\% - note such spindles typically failed to migrate to the oocyte cortex), a spindle-like structures with mis-aligned or dispersed chromosomes (-PB1 +spindle defect; 22.9\%) or a non-nuclear membrane enveloped ball of chromatin (-PB1+ultra-condensed chromatin; 25.7\%), collectively indicative of severely impaired segregation of bivalent chromosomes in a defective meiosis I (Fig. 7e). Despite, permitting assayed GV oocytes to meiotically mature for 18 hours, we wanted to confirm if the observed \textit{Wwc2} knockdown phenotypes would persist or resolve if provided with increased time (possibly indicative of delayed rather than blocked meiotic progression). We repeated the \textit{Wwc2} knockdown experiment allowing an extended IVM period of 24 hours. However, no increased frequency of MII arrested oocyte generation was observed; rather \textit{Wwc2} mRNA depleted oocytes either remained blocked in meiosis I or unsuccessfully attempted to segregate their chromosomes, without undergoing cytokinesis (Fig. S7).

\textit{Wwc2} specific oocyte meiotic maturation phenotypes are associated with failed Aurora kinase-A (AURKA) activation.

It is reported that activated/phosphorylated Aurora kinase-A (p-AURKA: p-Thr288) is required to initiate acentriolar/ MTOC mediated spindle assembly in mouse oocytes and that \textit{Aurka} knockdown phenotypes resemble those observed here for \textit{Wwc2} knockdown \textsuperscript{21}. We therefore hypothesised the meiotic maturation phenotypes identified in \textit{Wwc2} mRNA depleted GV oocytes may be associated with impaired levels of activated p-AURKA. A western blot analysis of staged maturing oocytes confirmed characteristically low levels of p-AURKA in GV oocytes, that robustly increased with the appearance of the first meiotic spindle (during MI) and were maintained with the formation of the second meiotic spindle (in MII arrest; Fig. 8b & \textsuperscript{15,16}; a trend replicated in control siRNA microinjected GV (assayed after 18 hour incubation in IMBX) and MII (after 18 hours post-IBMX removal) oocytes (note, the specificity of the anti-p-AURKA antibody used was additionally confirmed; Fig. S8). However, in the \textit{Wwc2} siRNA microinjected group, we were not able to detect p-AURKA at either the equivalent GV or MII stages. Moreover, we were also unable to detect p-AURKA immuno-reactivity localised to the poles of any spindles or spindle-like structures formed in \textit{Wwc2} knockdown oocytes, in stark contrast to that observed on MII arrested spindles in controls (Fig. 8c); confirming a failure to activate AURKA under conditions associated with profound meiotic oocyte maturation defects. We next employed the \textit{HA-Wwc2} rescue mRNA construct in an attempt to reverse the observed \textit{Wwc2} knockdown oocyte maturation phenotypes. Using a similar IVM assay (but assaying at multiple time-points), we found that co-microinjection of \textit{HA-Wwc2} mRNA and \textit{Wwc2} siRNA was able to robustly rescue the maturation phenotypes caused by \textit{Wwc2} siRNA microinjection alone (\textit{i.e.} MII arrested oocytes at 16 hours post-IBMX; control siRNA – 81.8\%, \textit{Wwc2} siRNA - 8.7\% & \textit{HA-Wwc2} mRNA plus \textit{Wwc2} siRNA – 76.0\%); moreover the progression...
of the rescue through recognisable maturation stages was in-step with that of control siRNA microinjected oocytes (as measured at 1, 6, 12 and 16 hours post-IBMX removal; Fig. 8d). Consistently we also found co-microinjection of the rescue *HA*-*Wwc2* mRNA construct was coincident with reappearance of activated p-AURKA protein (Fig. 8e), that was also detected in proximity to forming or matured MI/MII stage meiotic spindles (Fig. S9). Collectively, these data confirm a novel role for *Wwc2* in regulating appropriate bivalent chromosome segregation during mouse primary oocyte meiotic maturation that is strongly associated with activation of the acentriolar/MTOC mediated spindle forming meiotic/mitotic kinase AURKA.
DISCUSSION

In the current study, we have identified Wwc2, the sole murine WWC-domain containing paralog of the recognised Hippo-pathway activator Kibra\(^{56,57,58,59,60}\), as a novel mitotic regulator of preimplantation mouse embryo cell cleavage divisions (Figs. 1-3); plus a similar role in primary mouse oocyte meiotic maturation (Fig. 7), predicated on appropriate activation/phosphorylation of the cell-cycle kinase AURKA (Figs. 8 and S9). Additionally, we have shown Wwc2 knockdown cell clones are compromised in ultimately contributing to the specified EPI lineage, by the late blastocyst stage, being prone to apoptosis or disproportionate contribution to PrE (Figs. 5 and S4). Thus, collectively these data implicate at least two functional roles for Wwc2 during early mouse development/reproduction; i) spatial and temporal regulation of correct cell division spindle apparatus formation and function in both meiotic primary oocytes or early mitotic embryonic blastomeres (each paradigms of typical and atypical, acentrosomal/acentriolar cell division, relative to other mammalian species, respectively \(^{5,6,8,9,10,11}\)), and ii) affecting individual blastomere pluripotency and differentiation, particularly with respect to EPI and PrE derivation in the ICM of late mouse blastocysts (E4.5) (Figs. 5 and S4); however, the full extent of such a cell fate related role, remains difficult to experimentally dissect, given the described cell division defects evident as early as the 8-cell (E2.5) stage after Wwc2 knockdown.

Relating to cell fate regulation, the attainment of naïve pluripotency within specified EPI cells of the late (E4.5) mouse blastocyst has recently been shown to be regulated via a heterogeneous mechanism of activated TEAD1-mediated transcription (caused by YAP relocalisation from the cytoplasm to the nucleus), that promotes ICM cell/EPI progenitor competition and elimination (by apoptosis)\(^{54}\). This contrasts with earlier specification and segregation of outer TE from founder ICM populations, prior to the blastocyst stage, whereby activated Hippo-signalling and YAP nuclear exclusion is required for inner-cells to actively retain pluripotency (e.g. express Sox2) and resist TE differentiation \(^{49}\). Hence, active Hippo-signalling seems to both support initial pluripotency in ICM founders (i.e. confirmed - see \(^{49}\)) but presumably needs to be suppressed in EPI progenitors to promote naïve pluripotency (i.e. to permit nuclear YAP translocation, inferred from \(^{54}\)). Our data, although compounded by the additional cell division phenotypes, suggests Wwc2 (as a paralog of the Hippo-activator Kibra) also contributes to germane Hippo-signalling regulation. For example, relating to late blastocyst (E4.5) stage EPI specification, we find ICM-residing Wwc2 knockdown clones, although fewer than their non-microinjected clone counterparts, do not display the same equal contribution to populate the EPI (NANOG+/SOX2+) and PrE (GATA4+) as observed in the non-specific siRNA microinjected clones of control embryos (Figs. 5 and S4). Indeed, such clones statistically favour PrE over EPI contribution, indicative of compromised pluripotent potential. Whether this is due to compromised Hippo-signalling activation after initial internalisation prior to blastocyst formation, and/or relates to relative fitness deficiencies affecting their ability to successfully compete to colonise specified EPI, is uncertain. However, an increased apoptotic ICM cell number within Wwc2 knockdown clones, often marked by detectable SOX2 protein expression (but never GATA4 immunofluorescence), observed in late (E4.5) blastocysts (Fig. 5b & c) is consistent with their competitive elimination. Although, the possibility remains the apparent lack of EPI fitness, associated with Wwc2 knockdown, is caused by an initial pluripotency deficit in ICM founders. Nevertheless, our analysis of early stage (E3.5) clonal Wwc2 knockdown blastocysts, suggests the establishment of differential Hippo-signalling between TE (i.e. suppression) and ICM (i.e. activation) was largely intact (Figs. 4 and S3; i.e. CDX2, PARD6B,
CDH1 and YAP immuno-fluorescent staining). Although, the comparatively reduced (or sometimes absent) expression of outer-cell CDX2 protein, plus less efficient YAP protein nuclear exclusion in inner-cells, in \textit{Wwc2}\textsuperscript{knockdown} clones (compared with sister non-microinjected clones within the same embryo or non-specific siRNA microinjected clones in control embryos; Figs. 4 and S3) implies some degree of mild early onset mild Hippo-signalling defect; potentially becoming more manifest/relevant during blastocyst maturation/EPI specification. The relatively mild early cell fate effects may also reflect functional redundancy between WWC2 and KIBRA, whereby potential major Hippo-signalling defects caused by specific \textit{Wwc2} knockdown are compensated by continued and sufficient expression of \textit{Kibra}; indeed, we demonstrated robust \textit{Wwc2} knockdown does not affect \textit{Kibra} mRNA levels (Fig. 1 and S1). Support for functional redundancy between WWC-domain proteins paralogs has been demonstrated in human cells (relating to KIBRA, WWC2 and WWC3 \textsuperscript{60}) and genetic ablation of the mouse \textit{Kibra} gene is developmentally viable (with adult mice only exhibiting learning and memory deficits \textsuperscript{71}). Interestingly, our initial clonal dsRNA mediated knockdown of \textit{Kibra} and \textit{Wwc2} expression, either in isolation or combination (Fig.1), demonstrated robust down-regulation of \textit{Kibra} alone did not impair formation of cavitated late (E4.5) blastocysts (comprising equal cell numbers to control dsRNA groups), suggesting loss of the \textit{Kibra} paralog could be tolerated, potentially due to continued \textit{Wwc2} expression. However, sole \textit{Wwc2} knockdown caused the profound defects present in this study, despite continued expression of \textit{Kibra}, and were not exacerbated by co-down regulation of \textit{Kibra}. Hence, if KIBRA and WWC2 proteins share functional redundancy during mouse preimplantation development, it is likely only in relation to Hippo-signalling regulation and not applicable to the described cell division roles of WWC2.

As described, siRNA mediated \textit{Wwc2} knockdown causes cell autonomous deficits in total preimplantation mouse embryo cell number (particularly affecting inner-cell populations), by the 16-cell (E3.0) stage (Figs. 2, 3 and S2). Why reduced embryo cell numbers are manifest comparatively late, given initial microinjection is performed 2-cell (E1.5) stage embryos is unclear. Possibly, all subsequent cell-cycles in \textit{Wwc2} knockdown clones are elongated in a manner that is only revealed on the whole embryo level by the 16-cell (E3.0) stage, implicating \textit{Wwc2} as a regulator of the atypically long cell-cycles of preimplantation mouse/mammalian embryo blastomeres (reviewed \textsuperscript{37}). Alternatively, it may reflect the developmental point maternally provided WWC2 protein, unaffected by siRNA, is functionally depleted. However, it impossible here to directly probe WWC2 protein expression, due to a lack of available antibodies. Although, we did confirm the abundant presence of poly-adenylated maternal \textit{Wwc2} mRNAs in both primary (associated with translating polysomes) and secondary mouse oocytes and zygotes (Fig. 7a & b), that coupled with the functional role in meiotic maturation (Figs. 7, 8, S7 and S9), strongly suggest maternal WWC2 protein inheritance in early cleavage stage embryos. The appearance of cell number deficits from the 16-cell (E3.0) stage also coincides with the first spatial segregation of blastomeres \textit{(i.e.} outer- and inner- \textit{positions}) and polarity/position dependant differential Hippo-signalling establishment and TE versus ICM cell fate derivation \textsuperscript{49}. As \textit{Wwc2} is a paralog of the confirmed Hippo-activator \textit{Kibra}\textsuperscript{56, 57, 58, 59, 60}, it is tempting to speculate the developmental timing of the cell number phenotype is particularly relevant. However, our experimental data suggest Hippo-signalling at this time \textit{(i.e.} prior to blastocyst formation) is at most mildly affected by \textit{Wwc2} knockdown. Additionally, the onset of individual nuclear morphological phenotypes, indicative of defective cell division/cytokinesis \textit{(e.g.} mid-body association, bi-/multi-/micro-nucleation; Fig. 3), were already evident by the 8-cell (E2.5) stage. Moreover, whilst average cell number was impaired in \textit{Wwc2} knockdown embryos after the 8-cell
(E2.5) stage, it did not significantly increase again until the mid-blastocyst (E4.0) stage (remaining around 8-10 cells after global 
Wwc2 knockdown, through the 16-/32-cell, E3.0/E3.5, stage equivalents; Fig. 2). This is significant as the mid-blastocyst (E4.0) stage reflects the developmental point at which the atypically acentrosomal/acentriolar cell cleavage divisions of mouse embryos, in comparison with most other mammalian species, reverts back to centrosomal control, after de novo centrosomes synthesis 11 (although it could also reflect the waning effect of Wwc2 specific siRNA). Therefore, we propose our data demonstrate a role for WWC2 in regulating acentrosomal cell division in preimplantation stage mouse blastomeres, prior to the mid-blastocyst (E4.0) stage. Moreover, that WWC2 is a regulator of mitotic spindle formation:functional dynamics, as reflected in phenotypic nuclear morphologies observed after Wwc2 knockdown (Fig. 3). Supporting this hypothesis, we readily detected recombinant HA-WWC2 protein (derived from microinjected siRNA resistant recombinant mRNA, used to successfully elicit phenotypic rescue of Wwc2 knockdown phenotypes – Figs. 6 and S5) in confirmed mitotic mid-bodies up until the early blastocyst (E3.5) stage but much less easily by the late blastocyst (E4.5) stage (Figs. 6 and S6); i.e. subsequent to de novo centrosome synthesis 11. The hypothesis is further substantiated by our data relating to primary oocyte maturation, in which GV stage oocytes depleted of Wwc2 transcripts are unable, again in the absence of centrosomes 5, 6, 8, 9, 10, to complete meiosis I and present with multiple spindle defects associated with failed activation/phosphorylation of the key cell-cycle kinase, AURKA (Figs. 7, 8, S7 and S9). The importance of AURK activity during early mouse embryo cell cleavage has been reported, whereby clonal siRNA mediated downregulation of Aurkb and Aurkc expression respectively increases or decreases the rate of mitotic cell division (Aurkb and Aurkc over-expression exhibit the opposing effects). Indeed, the reported cell number deficits associated with Aurkc down regulation, strongly resemble those related to Wwc2. Increased Aurkc expression was also shown to potentiate pluripotent gene expression, whilst enhanced Aurkc expression impaired nuclear retention of recombinant photostabilised-Oct4 reporter protein 14. It will be interesting to functionally probe any potential link between WWC2 and AURKB/AURKC, in the context of regulating pluripotency and Hippo-pathway regulation, in future studies.

The first report to implicate WWC-domain containing proteins in mammalian Hippo-signalling, demonstrated human KIBRA (in HEK293T cells) binding to, and stimulating the phosphorylation/activation, of LATS1/2, leading to subsequent phosphorylation of YAP 59. The same authors later described mitosis specific KIBRA phosphorylation, catalysed by AURKA and/or AURKB at a highly conserved AURK consensus/target motif (centred on Ser539), that could be antagonised by the mitotic phosphatase PP1. Moreover, they reported over-expression of a KIBRA mutant (S539A), promotes precocious M-phase exit in a model of SAC arrest, despite continued activation of cyclin dependent kinase, CDK1 72. Thus, implicating KIBRA as an important molecular component of M-phase progression/dynamics. Subsequently, the same group demonstrated KIBRA is required to fully activate/phosphorylate AURKA, that in turn phosphorylates/activates LATS2, ensuring its appropriate centrosomal localisation. Moreover, that siRNA mediated KIBRA transcript knockdown caused profound spindle defects, lagging chromosomes and micronuclei formation, reminiscent of Wwc2 knockdown-related phenotypes, plus centrosomal fragmentation in human MCF7 and HeLa cell line models 73; typical of direct LATS2 disruption, itself known to promote centrosomal structural integrity 74 and accumulation of gamma-tubulin required for initiating mitotic spindle assembly 75. Interestingly, we have now confirmed the presence of the same AURK consensus motifs in both murine KIBRA and WWC2 proteins (Fig. S10). Therefore, it is tempting to speculate the Wwc2 knockdown phenotypes we observe in preimplantation stage mouse embryos may be mechanistically related to those previously
described for human KIBRA, although with the caveat they function on the level of MTOC regulation, in the absence of centrosomes/centrioles [11]. For example, targeted genetic ablations of the Lats1 and Lats2 genes in mice are not described in association with the preimplantation embryo cell division/cell number deficits typical of Wwc2 knockdown, nor those described phenotypes above in relation to other models of mitosis. Rather, Lats1−/−/Lats2−/− early blastocysts (E3.5) only present with ectopic nuclear YAP expression within ICM cells [40]. Therefore, not all insights will be directly transferable. However, despite the lack of centrosomes in pre-mid-blastocyst stage mouse embryos, classical key centrosome regulators are expressed and been shown to have functional cell division roles. 

For example, genetic knockout of the M-phase cell-cycle regulator, Polo-like kinase 1 (Plk1), known to cooperate with AURKA within centrosomes to mediated bi-polar spindle formation [76], interestingly arrests at the same 8-cell (E2.5) developmental stage at which embryos subject to global Wwc2 knockdown first present with cell division defects (Figs. 2 and 3) [77]. Moreover, a critical role for the related Plk4 gene, itself recognised as a key regulator of centriole formation/duplication [78, 79], has been demonstrated during acentrosomal mouse blastomere division.

Accordingly, active PLK4 protein localises within the coalescing MTOCs forming around condensed chromatin and in cooperation with the centrosomal protein CEP152, promotes microtubule nucleation and bi-polar spindle formation and experimental depletion of active PLK4 results in the formation of mono-polar microtubule asters [22].

Hence, it will be interesting to further investigate the identified role of Wwc2 during early embryo acentrosomal cell division, through the prism of such pre-existing characterised, and MTOC-related/regulating, molecular components.

Furthermore, to test applicability of such potentially identified mechanisms within the conceptually similar paradigm of acentrosomal meiotic divisions of the maturing primary oocyte (also identified here as under control of Wwc2). Indeed, a role for PLK4 in mediating acentriolar spindle assembly in mouse oocytes, was recently described, whereby MTOC resident PLK4, in cooperation with AURKA, participates in initiating microtubule nucleation around condensing chromosome bivalents upon meiosis re-entry. However, unlike in mitotic blastomeres, functional inhibition of PLK4 does not completely block bi-polar spindle formation but rather extends assembly completion time. Moreover, the oocyte MTOC related roles of PLK4 and AURKA are only partially overlapping, with AURKA contribution appearing to be dominant (e.g. catalytically targeting the microtubule minus end stabiliser TACC3) [21]. Interestingly, the identified MTOC mediated spindle formation role of PLK4/AURKA is distinct from the co-existing mechanism of chromosome derived RAN-GTP gradient driven microtubule stabilisation in oocytes (as reviewed [5, 6, 8, 9, 10]). Clearly, in the context of Wwc2 knockdown oocyte maturation phenotypes described here (that include failed AURKA phosphorylation/activation), potential links between described PLK4/AURKA phenotypes and WWC2 merit further investigation. They are also consistent with reports implicating AURKA in the regulation of MTOC dynamics and microtubule spindle nucleation [15, 17, 18, 19]. However, importantly the described inhibition of PLK4 was associated with eventual polar body formation [21], unlike after Wwc2 knockdown (even after an extended IVM incubation time – Fig. S7), indicating that whilst meiotic spindle assembly is affected/impaired by Wwc2 knockdown (illustrated by a 25.7% incidence of spindle formation failure and a further 22.9% of oocytes exhibiting defective spindles - Fig. 7e), other impediments to successful meiotic maturation must exist (potentially relating, although not necessarily limited, to spindle migration, SAC, cytokines etc.). The comparative spread of observed meiotic maturation failure phenotypes associated with Wwc2 knockdown (i.e. failed spindle formation, defective spindles or persistent/ non-dividing MI spindles – Fig. 7e) also suggests a degree of functional redundancy may exist in response
to loss of WWC2 protein; for example, potentially related to the compensatory abilities of three expressed AURK paralogs (*i.e.* Aurka, Aurkb and Aurke), as demonstrated in combinatorial genetic ablations in mouse oocytes. In conclusion, we have identified the ill-characterised WWC-domain containing mouse gene *Wwc2* as an important cell-cycle regulator in both preimplantation mouse embryo blastomeres and maturing primary oocytes; both paradigms of acentrosomal cell division. Additionally, we have uncovered evidence for *Wwc2*, as a paralog of the classically described Hippo-signalling activator *Kibra*, in participating in cell fate regulation in regard to blastocyst ICM pluripotency. Despite the compounding nature of the two identified *Wwc2* knockdown phenotypes, it will be of great interest to experimentally determine the key molecular interactions and further understand the novel roles played by WWC2 protein, in both mouse preimplantation embryo cell fate and mitosis and maturing primary oocyte meiosis.
METHODS

Mouse/mouse embryo related experimental procedures were approved and licensed by the local ethics committee of the Biology Centre (in České Budějovice) of the Czech Academy of Sciences and by the responsible committee of the Czech Academy of Sciences at the national level, in accordance with Czech and European Union law.

Embryo and oocyte culture and microinjection.

2-cell stage (E1.5) embryo collection, from super-ovulated and mated 10 week old F1 hybrid (C57Bl6 x CBA/W) female mice, and in vitro culture in mineral oil covered drops (~20 µL/ ~15 embryos) of commercial KSOM (EmbryoMax® KSOM without Phenol Red; Merck, MR-020P-5F), was conducted as previously described 48. Germinal vesicle (GV) stage primary oocytes were mechanically recovered from mature Graafian follicles of dissected ovaries of dams after PMSG (pregnant mare serum gonadotrophin) hormone stimulation (7.5U intraperitoneal injection of F1 hybrid strain, 48 hours prior). Recovered oocytes were placed into pre-warmed (37°C) drops (~20 µl/ ~15 oocytes) of commercial CZB media (EmbryoMax® CZB media with Phenol Red; Merck, MR-019-D) containing 100 µM IBMX (3-isobutyl-1-methylxanthine, a cAMP/cGMP phosphodiesterase inhibitor that maintains GV meiosis I arrest ), overlaid with mineral oil and incubated (37°C/ 5% CO₂) for 2 hours prior to microinjection.

Individual blastomere dsRNA, siRNA or recombinant mRNA microinjections (or combinations thereof), plus post-microinjection culture protocols, were performed on 2-cell stage embryos (in either one or both blastomeres) according to defined protocols 80, using apparatus formerly described 48. Recovered GV staged oocytes were microinjected using a minimally adapted protocol; namely oocytes were microinjected on 37°C heated stage in concaved glass microscope slides filled with CZB media (+ IBMX inhibitor, 100 µM), overlaid with mineral oil. Post-microinjection, GV oocytes were returned to the incubator for another 18 hours (to permit microinjected siRNA mediated gene knockdown or mRNA expression; CZB +IBMX media) then transferred to fresh/pre-equilibrated CZB media drops lacking IBMX, to induce resumption of meiosis I and in vitro maturation. Cultured (and microinjected) embryos and oocytes were assayed at various developmental points, as dictated by individual experiments. As indicated, rhodamine-conjugated dextran beads (RDBs; 1 µg/ µl – ThermoFisher Scientific, D1818) were co-microinjected to confirm successful blastomere/oocyte microinjection; a summary of the origin and concentrations of all microinjected RNA species is given in supplementary methods tables SM1. Non-microinjected embryos, or oocytes, (2-3 per microinjection experiment, per plate) served as culture sentinels to confirm successful in vitro embryo development/oocyte maturation. In cases of AURKA chemical inhibition (to confirm specificity of anti-p-AURKA antiser – Fig. S8), recovered GV oocytes were transferred from IMBX containing CZB media to pre-equilibrated CZB media drops lacking IBMX but containing the specific AURKA inhibitor, MLN8237 (1µM; Selleckchem, S1133).

dsRNA and recombinant mRNA synthesis.

Specific long dsRNA molecules targeting coding regions of mouse Kibra and Wwc2 derived transcripts (plus negative control GFP) were designed (and in silico validated using online E-RNAi resource 85) and synthesised.
Briefly, gene specific PCR primer pairs, incorporating 5’- T7-derived RNA polymerase promoters and spanning designed dsRNA complementary sequence, were used to derive *in vitro* transcription (IVM) template, using mouse blastocyst (E3.5) cDNA as a template (or plasmid DNA for GFP). After agarose gel verification, the double stranded DNA templates were used in preparatory IVM reactions, incorporating DNaseI and single-stranded RNase treatment (MEGAscript T7; ThermoFisher Scientific, AMB13345), to generate dsRNA. The integrity of derived *Kibra-*Wwc2- and *GFP*-dsRNAs was confirmed by non-denaturing gel electrophoresis and quantified (NanoDrop). The PCR primer sequences used are provided in supplementary methods table SM2.

Microinjected mRNA constructs were derived using commercially available IVT reaction kit (mMACHINE T3; ThermoFisher Scientific, AM1348), in which restriction enzyme linearized (using *SfiI*) plasmid DNA was used as template (2 µg) as follows; i. C-terminal GFP-fusion *Gap43* mRNA plasma membrane marker, from pRN3P-C-term-GFP-Gap43, ii. C-terminal RFP-fusion histone H2B mRNA (from pRN3-C-term-RFP-Hist1h2bb, derived in this study) and iii. siRNA-resistant N-terminal HA-tagged *Wwc2* mRNA (from pRN3P-N-term-HA-siRNAres-Wwc2, derived here). All synthesised mRNAs were subject to post-synthesis 3’ poly-adenylation tailing (PolyA Tailing Kit - ThermoFisher Scientific, AM1350), confirmed by denaturing gel electrophoresis and quantified (NanoDrop).

**Recombinant plasmid construct generation.**

Four recombinant plasmids (required for IVT) were generated, using standard molecular biological protocols. RFP-histone H2B fusion protein (pRN3-C-term-RFP-Hist1h2bb) were derived by in frame cloning of a high-fidelity PCR amplified cDNA (using Phusion® DNA polymerase, New England BioLabs, M05305) encoding histone H2B (*Hist1h2bb*, lacking endogenous start and stop codons), flanked by oligonucleotide introduced restriction sites (*NheI*), into the RNA transcription cassette plasmid pRN3-insert-RFP variant (vector encodes required start and stop codons). Thus, generating required H2B-RFP fusion reporter, downstream of vector encoded T3 RNA polymerase promoter (for IVT) and flanked by UTRs from the frog β-globin gene. The IVT plasmid encoding the siRNA-resistant N-terminal HA-tagged *Wwc2* mRNA (pRN3P-N-term-HA-siRNAres-Wwc2) was created from an annotated full-length Riken mouse *Wwc2* cDNA clone (clone ID: M5C1098O04, Source Biosciences) by deriving a high fidelity PCR product, with oligo introduced 5’ (*SpeI*) and 3’ (*NotI*) restriction sites and an in frame N-terminal HA-epitope tag, and `TA cloned’ (after addition of 3’ adenine nucleotide overhangs) it into pGEM®-T-Easy plasmid vector (Promega, A1360). The derived plasmid was site mutagenised (commercial service; EuroFins) to alter the nucleotide (but not amino acid codon) sequence of the siRNA (utilised in this study) recognition motif; details in supplementary figure S5. The recombinant gene sequence was sub-cloned, using introduced *SpeI* and *NotI* restriction sites, into the multiple cloning site of the RNA transcription cassette plasmid pRN3P to derive IVT competent pRN3P-N-term-HA-siRNAres-Wwc2. All four derived plasmids were sequence verified and the PCR primers (or relevant details) used to generate the required inserts are described in supplementary methods table SM3.

**Q-RT-PCR**

Per experimental condition, total RNA was extracted from ~30 (microinjected) mouse embryos (oocytes) *in vitro* cultured to the desired developmental stage, using PicoPure RNA isolation kit (Arcturus Biosciences/ThermoFisher Scientific, KIT0204), as instructed. Eluted RNA (10 µL) was DNaseI treated (DNA-free kit;
ThermoFisher Scientific, AM1906) and used to derive cDNA (30 µL), either via oligo-dT (embryos and oocyte) or random hexamer (oocytes only) priming (Superscript-III Reverse Transcriptase; ThermoFisher Scientific, 18080085). 0.5 µl of diluted template cDNA (1:3, nuclease-free water) per real-time PCR reaction (10 µl – SYBR Green PCR kit, Qiagen, 204143) was used to assay specific transcript abundance (CFX96 Real-Time System, BioRad). Kibra and Wwc2 transcript levels were internally normalised against those for Tbp (TATA-binding protein) housekeeping gene and fold changes (±s.e.m.) derived using the ΔΔCt method 84. A minimum of two biological replicates of at least three technical replicates were employed; specific gene oligo primer sequences (final reaction conc. 400 nM) are in supplementary methods table SM4.

Immuno-fluorescent staining and confocal microscopy imaging.

In vitro cultured (microinjected) embryos/oocytes were fixed (at required developmental stages) with 4% para-formaldehyde, immuno-fluorescently stained and imaged in complete z-series by confocal microscopy (using the FV10i confocal microscope, Olympus) as previously described 48; supplementary methods table SM5 summarises the identity and combinations (plus employed concentrations) of primary and fluorescently conjugated secondary antibodies used. Additionally, the majority of immuno-fluorescently stained embryos/oocytes were counterstained for DNA, using DAPI containing mounting solution (Vectashield plus DAPI, Vector Labs) and as indicated some embryo samples were also counterstained against filamentous-actin, using fluorescently-labelled rhodamine-conjugated phalloidin (ThermoFisher, R415 - described in 45).

Embryo/oocyte image analysis/ cell counting.

The contribution of each individual cell, per fixed embryo sample (in control or Kibra/Wwc2 gene RNAi knock-down conditions), to either; inner (entirely encapsulated) outer (whereby a cell retains a cell contactless domain) embryo compartments, emerging blastocyst cell lineages (defined by presence and/or absence of specific and stated lineage marker protein expression), cell populations defined by the presence of a defining intra-cellular feature (e.g. cell/nuclei morphological abnormalities, apoptosis or persistent mid-bodies) or presence within or outwith a microinjected clone (distinguishable by a fluorescent injection marker signal, i.e. RDBs or recombinant fluorescent fusion proteins) was determined by serial inspection of individual confocal micrograph z-sections of each embryo, using commercial Fluoview ver.1.7.a (Olympus), Imaris (Bitplane) and freely available ImageJ software. These calculated contributions were individually tabulated (see appropriate supplementary tables) and the mean of cells within defined sub-populations, plus standard error of means (mean ± s.e.m.) determined. The statistical significance between relevant experimental and control groups was determined using 2-tailed Student’s t-tests (experiment specific supplementary tables provide comprehensive summaries, plus all individual embryo derived data). Relating to oocytes, a similar approach was used to assay the mean frequencies by which maturing oocytes exhibit intra-cellular morphologies (revealed by combined immuno-fluorescent staining, i.e. α-TUBULIN and p-AURKA and microinjected fluorescent reporter protein expression i.e. RFP-histone H2B) indicative of appropriate or aberrant GV oocyte maturation (as categorised), at the indicated developmental time-point; similarly such data are summarised in relevant supplementary tables, including individual oocyte derived data.

Western blotting
A precise number of oocytes per comparable experimental condition (~15-30) were washed in phosphate buffer saline (PBS; Merck, P5493) containing polyvinyl alcohol (PVA; Merck, 341584), frozen in a residual volume at -80°C, before lysis, in 10 µl of 10x SDS reducing agent/loading buffer (NuPAGE buffer, ThermoFisher Scientific, NP 0004, ThermoFisher Scientific), by boiling at 100°C for 5 minutes. Loaded proteins were electrophoretically separated on gradient precast 4–12% SDS–PAGE gels (ThermoFisher Scientific, NP0323) and transferred to Immobilon P membranes (Merck group, IVPD00010) using a semi-dry blotting system (Biometra/ Analytik Jena) for 25 minutes at 5 mA/cm². Blotted membranes were blocked in 5% skimmed milk powder dissolved in 0.05% Tween-Tris pH 7.4 buffered saline (TTBS) for 1 hour, briefly rinsed in TTBS and then incubated overnight at 4°C in 1% milk/TTBS containing primary antibody. Membranes were washed in three changes of TTBS buffer (20 minutes each at room temperature) and horse-radish peroxidase conjugated secondary antibody added to the blot in 1% milk/TTBS, for 1 hour (room temperature). Immuno-detected proteins were visualized by chemiluminescent photographic film exposure (ECL kit; GE Life Sciences, RPN2232) and digitally scanned using a GS-800 calibrated densitometer (Bio-Rad Laboratories). Antibody stripped membrane blots were re-probed, for loading controls, in an identical manner. Supplementary methods tables SM6 details the utilised concentrations of the primary and peroxidase-conjugated secondary antibodies used.
ACKNOWLEDGEMENTS

The authors acknowledge the Animal Facility (Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic) for housing experimental mice, Marta Gajewska (Institute of Oncology, Warsaw, Poland) and Anna Piliszek (Institute of Genetics & Animal Breeding, Polish Academy of Sciences, Jastrzębiec, Poland) for providing the founder CBA/W mice used in this study, Martin Anger (Central European Institute of Technology (CEITEC)/ Veterinary Research Institute, Brno, Czech Republic) for valuable technical advice and discussions and Alena Krejčí (Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic) for advice and pooling resources. The research was supported by a Czech Science Foundation/ GA ČR grant (18-02891S) awarded to A.W.B. and a Grant Agency of the University of South Bohemia Ph.D. student award to G.V (GA JU: 015/2017/P).

AUTHOR CONTRIBUTION

A.W.B. & G.V. designed the experiments, participated in data analysis (plus A.Š.) and drafted the manuscript (plus A.Š.). G.V. performed all experiments and prepared samples for western blotting (performed by A.Š.). P.B. derived the recombinant DNA constructs and L.G. performed important ancillary experiments, ultimately not included in the final manuscript. A.W.B. coordinated the study. All authors approved the final version of the manuscript for publication.

AUTHOR DECLARATION

The authors declare no competing interests.
REFERENCES

1. Johnson MH. From mouse egg to mouse embryo: polarities, axes, and tissues. *Annu Rev Cell Dev Biol* **25**, 483-512 (2009).

2. Sanders JR, Jones KT. Regulation of the meiotic divisions of mammalian oocytes and eggs. *Biochem Soc Trans* **46**, 797-806 (2018).

3. Hassold T, Hunt P. To err (meiotically) is human: the genesis of human aneuploidy. *Nat Rev Genet* **2**, 280-291 (2001).

4. Nagaoka SI, Hassold TJ, Hunt PA. Human aneuploidy: mechanisms and new insights into an age-old problem. *Nat Rev Genet* **13**, 493-504 (2012).

5. Bennabi I, Terret ME, Verlhac MH. Meiotic spindle assembly and chromosome segregation in oocytes. *J Cell Biol* **215**, 611-619 (2016).

6. Namgoong S, Kim NH. Meiotic spindle formation in mammalian oocytes: implications for human infertility. *Biol Reprod* **98**, 153-161 (2018).

7. Mihajlovic AI, FitzHarris G. Segregating Chromosomes in the Mammalian Oocyte. *Curr Biol* **28**, R895-R907 (2018).

8. Mogessie B, Scheffler K, Schuh M. Assembly and Positioning of the Oocyte Meiotic Spindle. *Annu Rev Cell Dev Biol* **34**, 381-403 (2018).

9. Gruss OJ. Animal Female Meiosis: The Challenges of Eliminating Centrosomes. *Cells* **7**, (2018).

10. Severson AF, von Dassow G, Bowerman B. Oocyte Meiotic Spindle Assembly and Function. *Curr Top Dev Biol* **116**, 65-98 (2016).

11. Courtois A, Schuh M, Ellenberg J, Hiiragi T. The transition from meiotic to mitotic spindle assembly is gradual during early mammalian development. *J Cell Biol* **198**, 357-370 (2012).

12. Schatten H, Sun QY. The role of centrosomes in mammalian fertilization and its significance for ICSI. *Mol Hum Reprod* **15**, 531-538 (2009).

13. Sathananthan AH, *et al.* Centrioles in the beginning of human development. *Proc Natl Acad Sci U S A* **88**, 4806-4810 (1991).

14. Li W, *et al.* Differential regulation of H3S10 phosphorylation, mitosis progression and cell fate by Aurora Kinase B and C in mouse preimplantation embryos. *Protein Cell* **8**, 662-674 (2017).

15. Nguyen AL, Schindler K. Specialize and Divide (Twice): Functions of Three Aurora Kinase Homologs in Mammalian Oocyte Meiotic Maturation. *Trends Genet* **33**, 349-363 (2017).

16. Saskova A, Solc P, Baran V, Kubelka M, Schultz RM, Motlik J. Aurora kinase A controls meiosis I progression in mouse oocytes. *Cell Cycle* **7**, 2368-2376 (2008).

17. Nguyen AL, *et al.* Genetic Interactions between the Aurora Kinases Reveal New Requirements for AURKB and AURKCC during Oocyte Meiosis. *Curr Biol* **28**, 3458-3468 e3455 (2018).

18. Solc P, *et al.* Aurora kinase A drives MTOC biogenesis but does not trigger resumption of meiosis in mouse oocytes matured in vivo. *Biol Reprod* **87**, 85 (2012).
Swain JE, Ding J, Wu J, Smith GD. Regulation of spindle and chromatin dynamics during early and late stages of oocyte maturation by aurora kinases. *Mol Hum Reprod* **14**, 291-299 (2008).

Kovarikova V, Burkus J, Rehak P, Brzakova A, Solc P, Baran V. Aurora kinase A is essential for correct chromosome segregation in mouse zygote. *Zygote* **24**, 326-337 (2016).

Bury L, *et al.* Plk4 and Aurora A cooperate in the initiation of acentriolar spindle assembly in mammalian oocytes. *J Cell Biol* **216**, 3571-3590 (2017).

Coelho PA, *et al.* Spindle formation in the mouse embryo requires Plk4 in the absence of centrioles. *Dev Cell* **27**, 586-597 (2013).

Chazaud C, Yamanaka Y. Lineage specification in the mouse preimplantation embryo. *Development* **143**, 1063-1074 (2016).

Frum T, Ralston A. Cell signaling and transcription factors regulating cell fate during formation of the mouse blastocyst. *Trends Genet* **31**, 402-410 (2015).

Rossant J. Genetic Control of Early Cell Lineages in the Mammalian Embryo. *Annu Rev Genet* **52**, 185-201 (2018).

Rossant J. Making the Mouse Blastocyst: Past, Present, and Future. *Curr Top Dev Biol* **117**, 275-288 (2016).

White MD, Zenker J, Bissiere S, Plachta N. Instructions for Assembling the Early Mammalian Embryo. *Dev Cell* **45**, 667-679 (2018).

Mihajlovic AI, Bruce AW. The first cell-fate decision of mouse preimplantation embryo development: integrating cell position and polarity. *Open Biol* **7**, (2017).

O'Farrell PH. Growing an Embryo from a Single Cell: A Hurdle in Animal Life. *Cold Spring Harb Perspect Biol* **7**, (2015).

Bolton VN, Oades PJ, Johnson MH. The relationship between cleavage, DNA replication, and gene expression in the mouse 2-cell embryo. *J Embryol Exp Morphol* **79**, 139-163 (1984).

Howlett SK, Bolton VN. Sequence and regulation of morphological and molecular events during the first cell cycle of mouse embryogenesis. *J Embryol Exp Morphol* **87**, 175-206 (1985).

Krishna M, Generoso WM. Timing of sperm penetration, pronuclear formation, pronuclear DNA synthesis, and first cleavage in naturally ovulated mouse eggs. *J Exp Zool* **202**, 245-252 (1977).

Smith RK, Johnson MH. Analysis of the third and fourth cell cycles of mouse early development. *J Reprod Fertil* **76**, 393-399 (1986).

Parisi E, Filosa S, De Petrocellis B, Monroy A. The pattern of cell division in the early development of the sea urchin. *Paracentrotus lividus*. *Dev Biol* **65**, 38-49 (1978).

Newport J, Kirschner M. A major developmental transition in early *Xenopus* embryos: I. characterization and timing of cellular changes at the midblastula stage. *Cell* **30**, 675-686 (1982).

Foe VE, Alberts BM. Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in Drosophila embryogenesis. *J Cell Sci* **61**, 31-70 (1983).

O'Farrell PH, Stumpff J, Su TT. Embryonic cleavage cycles: how is a mouse like a fly? *Curr Biol* **14**, R35-45 (2004).
38. Davis JR, Tapon N. Hippo signalling during development. Development 146, (2019).
39. Leung CY, Zernicka-Goetz M. Angiomotin prevents pluripotent lineage differentiation in mouse embryos via Hippo pathway-dependent and -independent mechanisms. Nat Commun 4, 2251 (2013).
40. Nishioka N, et al. The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. Dev Cell 16, 398-410 (2009).
41. Cockburn K, Biechele S, Garner J, Rossant J. The Hippo pathway member Nf2 is required for inner cell mass specification. Curr Biol 23, 1195-1201 (2013).
42. Kono K, Tamashiro DA, Alarcon VB. Inhibition of Rho-ROCK signaling enhances ICM and suppresses TE characteristics through activation of Hippo signaling in the mouse blastocyst. Dev Biol 394, 142-155 (2014).
43. Alarcon VB. Cell polarity regulator PARD6B is essential for trophectoderm formation in the preimplantation mouse embryo. Biol Reprod 83, 347-358 (2010).
44. Hirate Y, et al. Polarity-dependent distribution of angiomotin localizes Hippo signaling in preimplantation embryos. Curr Biol 23, 1181-1194 (2013).
45. Mihajlovic AI, Bruce AW. Rho-associated protein kinase regulates subcellular localisation of Angiomotin and Hippo-signalling during preimplantation mouse embryo development. Reprod Biomed Online 33, 381-390 (2016).
46. Nishioka N, et al. Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. Mechanisms of development 125, 270-283 (2008).
47. Yagi R, et al. Transcription factor TEAD4 specifies the trophectoderm lineage at the beginning of mammalian development. Development 134, 3827-3836 (2007).
48. Mihajlovic AI, Thamodaran V, Bruce AW. The first two cell-fate decisions of preimplantation mouse embryo development are not functionally independent. Sci Rep 5, 15034 (2015).
49. Sasaki H. Roles and regulations of Hippo signaling during preimplantation mouse development. Dev Growth Differ 59, 12-20 (2017).
50. Hirate Y, Hirahara S, Inoue K, Kiyonari H, Niwa H, Sasaki H. Par-aPKC-dependent and -independent mechanisms cooperatively control cell polarity, Hippo signaling, and cell positioning in 16-cell stage mouse embryos. Dev Growth Differ 57, 544-556 (2015).
51. Wicklow E, et al. HIPPO pathway members restrict SOX2 to the inner cell mass where it promotes ICM fates in the mouse blastocyst. PLoS Genet 10, e1004618 (2014).
52. Frum T, Murphy TM, Ralston A. HIPPO signaling resolves embryonic cell fate conflicts during establishment of pluripotency in vivo. Elife 7, (2018).
53. Frum T, Watts JL, Ralston A. TEAD4, YAP1 and WWTR1 prevent the premature onset of pluripotency prior to the 16-cell stage. Development 146, (2019).
54. Hashimoto M, Sasaki H. Epiblast Formation by TEAD-YAP-Dependent Expression of Pluripotency Factors and Competitive Elimination of Unspecified Cells. Dev Cell 50, 139-154 e135 (2019).
55. Kremerskothen J, et al. Characterization of KIBRA, a novel WW domain-containing protein. *Biochem Biophys Res Commun* **300**, 862-867 (2003).

56. Genevet A, Wehr MC, Brain R, Thompson BJ, Tapon N. Kibra is a regulator of the Salvador/Warts/Hippo signaling network. *Dev Cell* **18**, 300-308 (2010).

57. Baumgartner R, Poernbacher I, Buse N, Hafen E, Stocker H. The WW domain protein Kibra acts upstream of Hippo in Drosophila. *Dev Cell* **18**, 309-316 (2010).

58. Yu J, Zheng Y, Dong J, Klusza S, Deng WM, Pan D. Kibra functions as a tumor suppressor protein that regulates Hippo signaling in conjunction with Merlin and Expanded. *Dev Cell* **18**, 288-299 (2010).

59. Xiao L, Chen Y, Ji M, Dong J. KIBRA regulates Hippo signaling activity via interactions with large tumor suppressor kinases. *J Biol Chem* **286**, 7788-7796 (2011).

60. Wennmann DO, et al. Evolutionary and molecular facts link the WWC protein family to Hippo signaling. *Mol Biol Evol* **31**, 1710-1723 (2014).

61. Lin Z, Yang Z, Xie R, Ji Z, Guan K, Zhang M. Decoding WW domain tandem-mediated target recognitions in tissue growth and cell polarity. *Elife* **8**, (2019).

62. Wang W, Li X, Huang J, Feng L, Dolinta KG, Chen J. Defining the protein-protein interaction network of the human hippo pathway. *Mol Cell Proteomics* **13**, 119-131 (2014).

63. Zhang Y, et al. WWC2 is an independent prognostic factor and prevents invasion via Hippo signalling in hepatocellular carcinoma. *J Cell Mol Med* **21**, 3718-3729 (2017).

64. Han Q, et al. WWC3 inhibits epithelial-mesenchymal transition of lung cancer by activating Hippo-YAP signaling. *Onco Targets Ther* **11**, 2581-2591 (2018).

65. Zenker J, et al. A microtubule-organizing center directing intracellular transport in the early mouse embryo. *Science* **357**, 925-928 (2017).

66. Johnson MH, Ziomek CA. The foundation of two distinct cell lineages within the mouse morula. *Cell* **24**, 71-80 (1981).

67. Strumpf D, et al. Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development* **132**, 2093-2102 (2005).

68. Terada Y, Tatsuka M, Suzuki F, Yasuda Y, Fujita S, Otsu M. AIM-1: a mammalian midbody-associated protein required for cytokinesis. *EMBO J* **17**, 667-676 (1998).

69. Salles FJ, Darrow AL, O'Connell ML, Strickland S. Isolation of novel murine maternal mRNAs regulated by cytoplasmic polyadenylation. *Genes Dev* **6**, 1202-1212 (1992).

70. Koncicka M, et al. Increased Expression of Maturation Promoting Factor Components Speeds Up Meiosis in Oocytes from Aged Females. *Int J Mol Sci* **19**, (2018).

71. Makuch L, et al. Regulation of AMPA receptor function by the human memory-associated gene KIBRA. *Neuron* **71**, 1022-1029 (2011).

72. Xiao L, et al. KIBRA protein phosphorylation is regulated by mitotic kinase aurora and protein phosphatase 1. *J Biol Chem* **286**, 36304-36315 (2011).
Zhang L, et al. KIBRA regulates aurora kinase activity and is required for precise chromosome alignment during mitosis. *J Biol Chem* **287**, 34069-34077 (2012).

Yabuta N, et al. Lats2 is an essential mitotic regulator required for the coordination of cell division. *J Biol Chem* **282**, 19259-19271 (2007).

Abe Y, Ohsugi M, Haraguchi K, Fujimoto J, Yamamoto T. LATS2-Ajuba complex regulates gamma-tubulin recruitment to centrosomes and spindle organization during mitosis. *FEBS Lett* **580**, 782-788 (2006).

Asteriti IA, De Mattia F, Guarguaglini G. Cross-Talk between AURKA and Plk1 in Mitotic Entry and Spindle Assembly. *Front Oncol* **5**, 283 (2015).

Lu LY, et al. Polo-like kinase 1 is essential for early embryonic development and tumor suppression. *Mol Cell Biol* **28**, 6870-6876 (2008).

Habedanck R, Stierhof YD, Wilkinson CJ, Nigg EA. The Polo kinase Plk4 functions in centriole duplication. *Nat Cell Biol* **7**, 1140-1146 (2005).

Bettencourt-Dias M, et al. SAK/PLK4 is required for centriole duplication and flagella development. *Curr Biol* **15**, 2199-2207 (2005).

Zernicka-Goetz M, et al. An indelible lineage marker for Xenopus using a mutated green fluorescent protein. *Development* **122**, 3719-3724 (1996).

Horn T, Boutros M. E-RNAi: a web application for the multi-species design of RNAi reagents--2010 update. *Nucleic Acids Res* **38**, W332-339 (2010).

Morris SA, Teo RT, Li H, Robson P, Glover DM, Zernicka-Goetz M. Origin and formation of the first two distinct cell types of the inner cell mass in the mouse embryo. *Proc Natl Acad Sci U S A* **107**, 6364-6369 (2010).

Lemaire P, Garrett N, Gurdon JB. Expression cloning of Siamois, a Xenopus homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* **81**, 85-94 (1995).

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif)* **25**, 402-408 (2001).
FIGURE LEGENDS

Figure 1: dsRNA mediated WWC-domain containing gene (Kibra and Wwc2) expression knockdown in preimplantation stage mouse embryos is associated with (Wwc2) reduced cell number. a) Experimental schema of Kibra and Wwc2 specific dsRNA (plus EGFP negative control) embryo microinjection mediated global mRNA knockdown, followed by in vitro culture to either 8-cell (E2.5) or 32-cell (E3.5) stage and Q-RT-PCR analysis (upper). Normalised mRNA expression of either Kibra or Wwc2 derived mRNAs after microinjection of stated dsRNA at the stated developmental stage (lower; error bars represent s.e.m. and n=3). b) Experimental schema, similar to a), describing clonal Kibra, Wwc2 or Kibra+Wwc2 knockdown (microinjection of one cell at the 2-cell/E1.5 stage) and total cell number assay (via fixed embryo DAPI staining) at the late blastocyst, >64-cell (E4.5) stage (upper). Average total cell numbers per embryo in each stated dsRNA microinjection group is shown (lower chart). c) Experimental strategy to knockdown Wwc2 expression (as in b) in a marked clone, by co-microinjection of Wwc2 dsRNA with rhodamine conjugated dextran microbeads (RDBs; fluorescent lineage marker) and assay clonal contribution at the 16-cell (E3.0) stage (upper). Average total cell number per clone (i.e. ‘Inj’, vs. ‘Non’) in Wwc2 or control dsRNA microinjection groups are shown, as determined by the relative position of individual cells within the embryo (i.e. encapsulated inside or with a cell contactless domain on the outside; lower chart). In panels b) & c) chart error bars represent s.e.m. and ‘n’ numbers are indicated; statistically significant differences (2-tailed students t-test) between the experimental microinjection groups (asterisks), or clones (‘Inj.’ vs. ‘Non’) within a group (double crosses), are highlighted with statistical confidence intervals of p<0.05 and p<0.005, as denoted by one or two significance markers, respectively; in panel b) a lack of significant difference between the compared groups is marked ‘n.s.’. Supplementary tables ST1 and ST2 summarise the statistical analysis and individual embryo data.

Figure 2: siRNA mediated global Wwc2 knockdown causes persistent preimplantation stage cell number deficits from the 8-cell stage onwards: a) Experimental design; non-specific control or Wwc2-specific siRNA were co-microinjected with RDBs, into both blastomeres at the 2-cell (E1.5) stage, and in vitro cultured, fixed and subject to total cell assay count (via DAPI and phallolidin staining) at the indicated developmental stage. A separate group of microinjected embryos at the 32-cell stage were subject to normalised Wwc2-specific Q-RTPCR to assay knockdown efficiency. b) Q-RTPCR data detailing the extent of endogenous Wwc2 expression knockdown caused by microinjected Wwc2-specific siRNA at the 32-cell (E3.5) stage (error bars denote s.e.m. of triplicate measurements, n=3). c) Average total cell number in control (blue bars) or Wwc2-specific (green bars) siRNA microinjected embryos at each indicated preimplantation developmental stages (the number of embryos in each assayed group is indicated). Errors represent s.e.m. and 2-tailed student t-test derived statistical significance between the control and Wwc2 knockdown groups, at defined developmental stages, indicated (** p<0.005). Supplementary tables ST3-ST8 summarise the statistical analysis and individual embryo data in each experimental group. Note, additional data describing the frequency of observable cell morphological/ division defects, obtained from this exact same dataset are presented in Fig. 3.

Figure 3: Global Wwc2 knockdown mediated cell numbers defects are coincident with accumulated cell morphologies consistent with defective cell division. a) Schematic of experimental design whereby 2-cell (E1.5)
stage embryos microinjected with either control or Wwc2-specific siRNA were in vitro cultured to the indicated developmental stage, fixed and assayed for total cell number (as described in Fig. 2) and the incidence of abnormal cell/embryo morphology indicative of aberrant cell division, as determined by DAPI and rhodamine conjugated phalloidin staining (note, data described in this figure are derived from same experiments as those described in Fig. 2). b) Exemplar confocal micrograph z-sections of Wwc2-specific siRNA microinjected embryos at the 32-cell (E3.5) stage, illustrating three distinct categories of observed phenotypic morphological defects; i. abnormal nuclear morphology (including chromatin mid body association - left), ii. cytokinesis defects defined by presence of two nuclei per cell (centre), iii. presence of multiple (i.e. >2) nuclei and/or micronuclei per cell (right) and iv. a composite of categorised defects highlighted by colour coded arrows in single embryo confocal z-section projection (green boarder). DAPI (white) and cortical F-actin (red); scale bar = 20 µm. c) Frequencies of each the observed categories of phenotypic/ morphological defect (i.e. micronuclei; blue, abnormal nuclei; yellow and failed cytokinesis; green) or a combination of at least two or more (red) in control siRNA (left) and Wwc2-specific siRNA (right) microinjected embryos, at the indicated developmental stage (numbers of embryos in each group is highlighted); data demonstrate defect incidence per embryo (in at least one constituent blastomere – upper row of charts) or on a per cell assayed basis (lower row of charts). Supplementary tables ST3-ST8 summarise the statistical analysis and individual embryo data in each experimental group.

Figure 4: Outer trophectoderm (TE) versus ICM derivation in clonal Wwc2 gene knockdown at the early blastocyst stage. a) Experimental strategy for Wwc2 expression knockdown in a marked clone (representing 50% of the embryo) by co-microinjection (in one blastomere of 2-cell/E1.5 stage embryos) of Wwc2 siRNA and GAP43-GFP mRNA (stable plasma membrane fluorescent lineage marker – ‘Inj.’) and an immunofluorescence (IF) based assay of clonal contribution (‘Inj’ vs. ‘Non’) to CDX2 (TE marker) positive or negative cells, plus outer and inner-cell embryo populations, at 32-cell (E3.5) stage (versus similar control siRNA microinjections). b) Exemplar confocal z-section micrographs of IF stained blastocysts (individual greyscale channels plus merged image; CDX2 is red) from control and Wwc2 siRNA microinjected groups. Note, white arrows and the yellow arrow head denote DAPI stained micronuclei and a bi-nucleated cell uniquely found within the Wwc2 siRNA microinjected clone, respectively. Similarly, blue and white asterisks denote respective low level (compared to non-injected clone) or undetectable outer-cell CDX2 protein expression in Wwc2 knockdown clones. The number of embryos in each group is provided; scale bar equals 20µm. c) Left - pie charts detailing the average distribution of detectable CDX2 protein expression between the control and Wwc2 siRNA groups, in both outer or ICM cells (note, pie chart areas for each spatial population are scaled to that summarising the whole embryo; similarly, the pie chart area describing the whole embryo in the Wwc2 siRNA condition is scaled to that in the control siRNA group). Right - average clonal contribution (‘Inj’ vs. ‘Non’) of outer and ICM cells in control (blue bars) and Wwc2 siRNA (green bars) microinjected embryos, irrespective of CDX2 expression. d) As in c) only describing the clonal contribution of CDX2 expressing (or not) cells to outer and ICM populations, as a fraction of the total sub-population size (left - pie charts) or in raw average cell number (right – bar charts) In panel c) and d) charts, errors represent s.e.m. and 2-tailed student t-test derived statistical significance between the control and Wwc2 knockdown groups (asterisks), or clones (‘Inj.’ vs ‘Non’) within a group (double crosses), are highlighted with statistical confidence intervals of p<0.05 and p<0.005, as denoted by
one or two significance markers, respectively. Supplementary tables ST14 summarise the statistical analysis and individual embryo data.

**Figure 5: Overall cell lineage derivation in clonal Wwc2 gene knockdown at the late blastocyst stage.**

- **a)** Experimental strategy for Wwc2 expression knockdown in a marked clone (representing 50% of the embryo) by co-microinjection (in one blastomere of 2-cell/E1.5 stage embryos) of Wwc2 siRNA and GAP43-GFP mRNA (stable plasma membrane fluorescent lineage marker – ‘Inj.’) and an IF based assay of clonal contribution (‘Inj.’ vs. ‘Non’) to blastocyst protein lineage marker (TE; CDX2, PrE; GATA4, EPI; NANOG or SOX2; assayed in combination – see below) expressing cells, plus outer and inner-cell embryo populations, at >64-cell, late blastocyst (E4.5) stage (versus similar control siRNA microinjections).
- **b)** Average number of apoptotic cells observed in control and Wwc2 siRNA microinjected embryo groups (averaged across all IF regimes – see below) between outer and inner cell populations and clones (‘Inj.’ vs. ‘Non’); errors represent s.e.m. and 2-tailed student t-test derived statistical significance between the control and Wwc2 knockdown groups (asterisks), or clones (‘Inj.’ vs ‘Non’) within a group (double crosses), are highlighted with statistical confidence intervals of p<0.05 and p<0.005, as denoted by one or two significance markers, respectively.
- **c)** Exemplar confocal z-section micrographs of IF stained blastocysts (individual greyscale channels plus merged image, in which assayed combinations of cell lineage markers are respectively coloured green and red; CDX2 & NANOG, GATA4 & NANOG and GATA4 and SOX2) from control and Wwc2 siRNA microinjected groups. Within the Wwc2 siRNA microinjected clone, yellow arrows denote apoptotic cell remnants, blue arrows interphase ICM cells that neither express GATA4 or NANOG, the yellow arrow head a bi-nucleated cell whereas blue or white asterisks highlight outer-cells with basal or undetectable CDX2 expression, respectively. The number of assayed embryos in each control (‘Con’) and Wwc2 siRNA (‘KD’) microinjection group is provided; scale bar equals 20µm.
- **d)** Pie charts detailing, in the left panel, the average distribution of detectable CDX2 and NANOG protein expression between the control and Wwc2 siRNA groups, in both outer or ICM cells (note, pie chart areas for each spatial population are scaled to that summarising the whole embryo; similarly the pie chart area describing the whole embryo in the Wwc2 siRNA condition is scaled to that in the control siRNA group). Central and right panels contain pie charts describing the clonal contribution of CDX2 and NANOG expressing (or not) cells to outer and ICM populations, as a fraction of the total sub-population size.
- **e)** As in d) only assaying NANOG (EPI) and GATA4 (PrE) expression.
- **f)** As in d) only assaying SOX2 (EPI) and GATA4 (PrE) expression. Note, supplementary figure S4 details the same data in raw average cell number format (i.e. as presented in Fig. 4, panel d) - bar charts on right) and supplementary tables ST15-ST17 summarise the statistical analysis and individual embryo data.

**Figure 6: Expression of siRNA resistant recombinant HA-Wwc2 mRNA rescues Wwc2 siRNA elicited cell number/division phenotypes.**

- **a)** Experimental strategy to assay potential phenotypic rescue of clonal Wwc2 expression knockdown by comparing co-microinjection (in one blastomere of 2-cell/E1.5 stage embryos) of GAP43-GFP mRNA (stable plasma membrane fluorescent lineage marker – ‘Inj.’) with either control siRNA, Wwc2 siRNA or Wwc2 siRNA + recombinant HA-Wwc2 mRNA (containing a N-terminal HA-epitope tag and point mutations conferring siRNA resistance – see supplementary figure S5) and assaying total, outer and inner-cell number at the 32- (E3.5) and >64-cell (late blastocyst/E4.5) stages.
- **b)** Average clonal contribution (‘Inj’ vs. ‘Non’) of outer and
Figure 7: **Wwc2 mRNA is required for mouse oocyte meiotic maturation.**

**a)** Published microarray (Wang et al., 2004, blue line) and generated Q-RT-PCR (bar charts) normalised Wwc2 mRNA expression in germinal vesicle and metaphase II arrested oocytes (GV and MII) and fertilised zygotes (Tbp normalised expression, relative to GV stage expression). Note, microarray data derived from oligo-dT primed reverse transcription and Q-RT-PCR from both oligo-dT (black bars) and random hexamer priming (red bars); error bars represent s.e.m. and n=3.

**b)** Taken from our published mRNA-Seq dataset of polysome-associated (red) and non-associated (blue) transcripts in *in vitro* maturing mouse oocytes (after germinal vesicle breakdown/ GVBD +3 hours) (Koncicka et al., 2018).

**c)** Experimental schema of *Wwc2* transcript knockdown in GV oocytes by microinjected *Wwc2* siRNA (plus control siRNA and non-microinjected controls), 18 hour incubation in IMBX containing media (to prevent GVBD), 16 hour *in vitro* oocyte maturation (IVM – minus IMBX treatment) and confocal microscopy/ Q-RT-PCR analysis; co-microinjected RDBs were used as injection control marker.

**d)** Q-RT-PCR data of normalised (against *Tbp* transcripts) *Wwc2* mRNA levels in control and *Wwc2* siRNA microinjected GV oocytes matured to the MII stage; expressed as percentage *Wwc2* mRNA knockdown, versus control (left), and normalised expression levels (right - error bars represent s.e.m. and n=3).

**e)** Charts (left) detailing successful maturation frequencies of control non-microinjected (Non-Inj), microinjected control siRNA (Con. siRNA) and microinjected *Wwc2* siRNA GV oocytes, to the MII stage or preceding phenotypic stages. Examples of successfully matured MII oocytes (note, extruded first polar body/PB1) and the quantified categorised phenotypes are shown as confocal z-section micrographs (right – α-Tubulin in green and DAPI DNA pseudo-coloured red; scale bar = 20 μm). Supplementary tables ST21 summarise the statistical analysis and individual oocyte data used to generate the figure.
Figure 8: *Wwc2* knockdown induced GV oocyte IVM phenotypes are associated with failed Aurora-A (AURKA) phosphorylation/activation; both rescuable by co-microinjection of siRNA resistant *HA-Wwc2* mRNA. a) Experimental schema of GV oocyte microinjection conditions; *i.e.* *Wwc2* siRNA or control siRNA alone or *Wwc2* siRNA + *HA-Wwc2* (siRNA resistant) mRNA, each co-microinjected with either RDBs (for western blot analysis) or RFP-H2B mRNA (IF – fluorescent marker). Microinjected (plus non-microinjected control) GV oocytes were incubated in IMBX containing media (18 hours - to prevent GVBD), subject to IVM (max. 16 hours – media minus IMBX) and processed for western blotting and IF at designated oocyte maturation time-points (assaying phospho-Aurora/ p-AURKA levels: p-Thr288) or assayed for developmental progression/*Wwc2* knockdown induced IVM phenotypes. b) Western blots of activated p-AURKA levels (plus GAPDH housekeeping control), at indicated stages of oocyte maturation (note, GV; +0 hours relative to IMBX washout, metaphase of meiosis I/ MI; +7 hours and MII; +16 hours), in either control or *Wwc2* siRNA microinjected, or non-microinjected conditions. c) Exemplar single confocal z-section IF micrographs of control (left) or *Wwc2* siRNA (right) IVM cultured microinjected oocytes to the MII stage (relative to control; 16 hours post-IMBMX) stained for p-AURKA (green) and α-TUBULIN (white) and labelled with RFP-H2B chromatin reporter (red); insets show zoomed region of meiotic spindle. *Wwc2* siRNA microinjection group example image is over-saturated compared to control (illustrating lack of spindle associated pAURKA); PB1 (control siRNA) denotes the extruded first polar body and the scale bars represents 20 µm. d) Charts detailing the maturation of control siRNA, *Wwc2* siRNA and *Wwc2* siRNA + *HA-Wwc2* mRNA microinjected GV oocytes at staged time-points after IBMX removal; as described by the percentage of oocytes at any of the stated IVM stages or categorised phenotypes (typically associated with *Wwc2* knockdown – see Fig. 7). e) Western blot of activated p-AURKA levels (plus GAPDH housekeeping control), at the MII equivalent stage (16 hours post-IVM), in either control siRNA, *Wwc2* siRNA or *Wwc2* siRNA + *HA-Wwc2* mRNA initially microinjected GV oocytes. Supplementary tables ST23-26 summarise the statistical analysis and individual oocyte data used to generate the figure.
Fig. 1

(a) 8-cell (E2.5) stage

- Q-RT PCR
- Assayed mRNA transcript
- Normalised expression (vs. Tbp)
- Kibra knockdown
- Wwc2 knockdown
- Assay cell number, blastomere position & clonal origin
- Clonal and relative spatial identity of assayed cells

(b) Assay total cell number

- dsRNA
- E1.5
- >64C
- E4.5

(c) Assay cell number, blastomere position & clonal origin

- Wwc2 or Control dsRNA (+RDBs)
- Inj.
- Non

- Total cell number per embryo (average)
- Total cell number per clone (average)

- Microinjected dsRNA condition
- Control (EGFP) n=17
- Kibra n=9
- Wwc2 n=10
- Wwc2 & Kibra n=5

- Inner cells
- Outer cells

- Control (EGFP) dsRNA
- Kibra dsRNA
- Wwc2 dsRNA

- n.s.
- **

- n=8
- n=10
**Fig. 2**

(a) Assay cell number and blastomere/embryo morphology

(b) Knocked-down/remaining mRNA

(c) Total cell number per embryo (average)

Assayed transcript

Wwc2 or Control siRNA (+RDBs)

E1.5

E2.0

E2.5

E3.0

E3.5

E4.0

E4.5

Q-RTPCR

Wwc2 Knocked-down/remaining mRNA

0%

20%

40%

60%

80%

100%

Assayed transcript

Control siRNA

Wwc2-specific siRNA

Assayed preimplantation embryo developmental stage

n=13 n=14

n=6 n=7

n=30 n=31

n=30 n=36

n=16 n=18

n=14 n=17
**Fig. 3**

**a)**

*Wwc2 or Control siRNA (+RDBs)*

Assay cell blastomere/embryo morphology

2C → 4C → 8C → 16C → 32C → 64C → >64C

E1.5 → E2.0 → E2.5 → E3.0 → E3.5 → E4.0 → E4.5

**b)**

Abnormal nuclear morphology

Cytokinesis defect

Multiple/micronuclei

Example nuclear defects in Wwc2 knockdown embryos

**c)**

| Phenotype                  | Observed | Unobserved |
|----------------------------|----------|------------|
| Presence of multiple/micronuclei |          |            |
| Abnormal nuclear morphology |          |            |
| Cytokinesis defect         |          |            |
| Amalgamated phenotypes     |          |            |

**Observed defects per embryo assayed (in at least one blastomere)**

| Control siRNA | Wwc2-specific siRNA |
|---------------|----------------------|
| E2.0          | n=13                 |
| E2.5          | n=6                  |
| E3.0          | n=30                 |
| E3.5          | n=30                 |
| E4.0          | n=16                 |
| E4.5          | n=14                 |

**Observed defects per blastomere/ cell assayed**

| Control siRNA | Wwc2-specific siRNA |
|---------------|----------------------|
|                |                      |
**Cell TE lineage (CDX2): IF staining.**

**Wwc2 or control siRNA (+GAP43-GFP mRNA)**

*E1.5 2C*

↓

*E3.5 32C*

**Control siRNA (n=14)**

**Wwc2-specific siRNA (n=18)**

*CDX2* & *GAP43-GFP merge*

**Fig. 4**

**a)**

**Experiment:**

- Microinjected (Inj.) clone
- Non microinjected (Non) clone

**b)**

**DAPI**

**GAP43-GFP**

**CDX2**

**c)**

**Whole Embryo**

- **Control siRNA**
  - Outer cells
  - Whole Embryo
  - Inner cells

- **Wwc2 siRNA**
  - Outer cells
  - Whole Embryo
  - Inner cells

**d)**

**Outer Cells**

- **Total Outer**
  - Control siRNA
  - Wwc2 siRNA

**Inner Cells**

- **Total Inner**
  - Control siRNA
  - Wwc2 siRNA

**Assayed cell identity**

- **Control siRNA**
  - Non microinjected (Non) clone
  - Microinjected (Inj.) clone

- **Wwc2 siRNA**
  - Non microinjected (Non) clone
  - Microinjected (Inj.) clone
**Fig. 5**

Wwc2 or control siRNA (+GAP43-GFP mRNA)

Cell lineage marker IF staining

### CDX2 & NANOG (Con/KD n=11/19)

- **Control siRNA**: DAPI, GAP43-GFP, CDX2 & NANOG
- **Wwc2 siRNA**: DAPI, GAP43-GFP, CDX2 & NANOG

### NANOG & GATA4 (Con/KD n=17/24)

- **Control siRNA**: DAPI, GAP43-GFP, GATA4 & NANOG
- **Wwc2 siRNA**: DAPI, GAP43-GFP, GATA4 & NANOG

### SOX2 & GATA4 (Con/KD n=16/20)

- **Control siRNA**: DAPI, GAP43-GFP, GATA4 & SOX2
- **Wwc2 siRNA**: DAPI, GAP43-GFP, GATA4 & SOX2

Clonal contribution OUTER cells

Clonal contribution INNER cells

DAPI

GAP43-GFP

CDX2

NANOG

GATA4

SOX2
**Fig. 6**

(a) IF staining (HA-epitope tag)

1. Control siRNA
2. Wwc2 siRNA
3. Wwc2 siRNA + siRNA rescue

(b) Experimental condition & assayed cell clone/position

|       | Early blastocyst (E3.5) | Late blastocyst (E4.5) |
|-------|-------------------------|------------------------|
| Non   | Inj.        | Outer cells | Inner cells | Inj. clone | Non clone | Outer cells & Inj. clone | Inner cells & Inj. clone | Normal nuclei | Abnormal nuclei |
|       | Average cell number | 0.0 | 2.0 | 4.0 | 6.0 | 8.0 | 10.0 | 12.0 | 14.0 | 16.0 |
|       |             | 5.0 | 10.0 | 15.0 | 20.0 | 25.0 | 30.0 | 35.0 | 40.0 |

(c) Whole embryo analysis

|       | Early blastocyst (E3.5) | Late blastocyst (E4.5) |
|-------|-------------------------|------------------------|
| Con. siRNA | Whole embryo analysis | Whole embryo analysis |
| Wwc2 siRNA | Outer cells | Inner cells | Inj. clone |
| Rescue    | Normal nuclei | Abnormal nuclei |

(d) Zoomed images

|       | Early blastocyst (E3.5) | Late blastocyst (E4.5) |
|-------|-------------------------|------------------------|
| DAPI | HA-tag | GAP43-GFP & HA-Tag | GAP43-GFP & HA-Tag & DAPI |
| Con. siRNA | DAPI | HA-tag | GAP43-GFP & HA-Tag | GAP43-GFP & HA-Tag & DAPI |
| Wwc2 siRNA | DAPI | HA-tag | GAP43-GFP & HA-Tag | GAP43-GFP & HA-Tag & DAPI |
| Rescue | DAPI | HA-tag | GAP43-GFP & HA-Tag | GAP43-GFP & HA-Tag & DAPI |
**Fig. 7**

**a)**

Graph showing normalised expression (Tbp) vs. GV.

**b)**

Bar chart showing Knockdown/remaining transcripts (%).

**c)**

Diagram of Assayed developmental time-point.

**d)**

Graph showing Normalised expression (Tbp) vs. Wwc2 mRNA.

**e)**

Graph showing Penetration of observed phenotypes (%).

**Experimental condition**

- Non
- Con
- Wwc2 siRNA

**Knockdown/remaining transcripts (%)**

- Non-associated transcripts
- Polysome-associated transcripts

**Assayed developmental time-point**

- GV
- MII
- Zygote

**Oocyte recovery** (M2 + IBMX)

**18 hour GVBD** inhibition (CZB + IBMX)

**16 hour in vitro maturation** (CZB - IBMX)

**Knockdown/remaining transcripts (%)**

- Wwc2 mRNA

**Penetration of observed phenotypes (%)**

- +PB1 +MII spindle
- -PB1 +MII spindle
- -PB1 +spindle defect/dispersed chromosomes
- -PB1 +ultra condensed chromatin

**Experimental condition**

- Non-inj
- Con siRNA
- Wwc2 siRNA

**Penetration of observed phenotypes (%)**

- n = 18
- n = 28
- n = 35
a) Wwc2 siRNA, control siRNA or Wwc2 siRNA + HA-Wwc2 (siRNA resistant) mRNA (+RDBs – WB or +RFP-H2B mRNA - IF)  

Non-microinjected control  

GV → GV → MII  

Detection of p-AURKA (p-Thr288) expression/activation  
Western blotting (WB)  
Immuno-fluorescence staining (IF)  

Oocyte recovery (M2 + IBMX)  
18 hour GVBD inhibition (CZB + IBMX)  
16 hour in vitro maturation (CZB - IBMX)  

b)  

| Treatment | GV | MII |
|-----------|----|-----|
| Cont. siRNA |   |     |
| Wwc2 siRNA |   |     |
| Cont. siRNA |   |     |
| Wwc2 siRNA |   |     |

Anti-p-AURKA (p-Thr288)  

Anti-GAPDH  

Wwc2 siRNA, control siRNA or Wwc2 siRNA + HA-Wwc2 (siRNA resistant) mRNA  

Dispersed chromosomes  
Ultra condensed chromosomes  

Cont. siRNA (MII)  
Wwc2 siRNA (~MI)  

Hours post-IBMX washout  

n=14  
n=15  
n=16  
n=22  
n=15  
n=18  
n=18  
n=23  

Wwc2 siRNA + HA-Wwc2 (siRNA res.) mRNA  

Anti-p-AURKA (p-Thr288)  
Anti-GAPDH  

+16 h IBMX washout (MII)