NIMA-related kinase 1 (NEK1) regulates meiosis I spindle assembly by altering the balance between α-Adducin and Myosin X

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Abstract

NIMA-related kinase 1 (NEK1) is a serine/threonine and tyrosine kinase that is highly expressed in mammalian germ cells. Mutations in Nek1 induce anemia, polycystic kidney and infertility. In this study we evaluated the role of NEK1 in meiotic spindle formation in both male and female gametes. Our results show that the lack of NEK1 provokes an abnormal organization of the meiosis I spindle characterized by elongated and/or multipolar spindles, and abnormal chromosome congression. The aberrant spindle structure is concomitant with the disruption in localization and protein levels of myosin X (MYO10) and α-adducin (ADD1), both of which are implicated in the regulation of spindle formation during mitosis. Interaction of ADD1 with MYO10 is dependent on phosphorylation, whereby phosphorylation of ADD1 enables its binding to MYO10 on mitotic spindles. Reduction in ADD1 protein in NEK1 mutant mice is associated with hyperphosphorylation of ADD1, thereby preventing the interaction with MYO10 during meiotic spindle formation. Our results reveal a novel regulatory role for NEK1 in the regulation of spindle architecture and function during meiosis.

Introduction

Meiosis is a specialized cell division characterized by a single round of DNA replication followed by two rounds of chromosome segregation, resulting in the formation of haploid gametes for sexual reproduction. At the first male meiotic division (MI), homologous (maternal and paternal) chromosomes must segregate equally into two daughter cells, each of which then undergoes a mitosis-like second meiotic division (MII) in which sister chromatids separate into the haploid gametes. In mammals, meiosis in males results in four haploid gametes, while meiosis in females results in one haploid gamete per meiosis, the remaining genetic material being distributed between two polar bodies. Regardless of the sex of the individual, in order to achieve accurate segregation at both divisions, tension must be established on the meiotic spindles and this is achieved by the formation of crossovers between homologous chromosomes in meiosis I, and by cohesion between sister chromatids in both meiosis I and meiosis II [1].
Cohesion is also necessary for sister kinetochore attachment to microtubules during meiotic spindle formation [2].

In eukaryotes, the microtubule spindle is the structure that orchestrates chromosome alignment and segregation during both mitotic and meiotic divisions [3]. In mitosis, the microtubules that compose the spindle are mostly nucleated from centrosomes, which act as a major microtubule organizing center (MTOC) [2–4]. Centrosomes are composed of pairs of centrioles surrounded by pericentriolar material (PCM) that possesses microtubule nucleation activity. When mitosis starts, centrosomes separate to opposite sides of the nuclear envelope, defining the poles of the spindle and allowing the bipolar spindle [2,4]. However, there is a sexual dimorphism during the meiosis, where the male spindle behaves like a mitotic spindle, while in oocytes, the spindle is formed without centrosomes [4,5]. Another important difference is the stringency of the spindle checkpoint in females, in which the oocyte still proceeds through the first meiotic division despite disruptions in the spindle and chromosome congression at metaphase plate [6]. Such differences in spindle dynamics in male and female mammals suggest that different regulatory proteins may predominate in each sex.

Several families of kinases such as Polo kinases, Aurora kinases and NEK kinases have been implicated in the regulation of cell cycle events in both mitosis and meiosis [7,8]. The founding member of the NEK family is the Aspergillus nidulans never-in-mitosis-gene-A (NIMA) protein [9]. NIMA is a serine/threonine kinase involved in the initiation of mitosis and in promoting chromosome condensation. Genetic ablation of NIMA results in cell cycle arrest at G2, while overexpression of NIMA leads to premature entry into mitosis [7]. In mammals, there are 11 orthologs of NIMA that comprise the NIMA-like (NEK) family of kinases. Nek1 is unique in that it encodes both the serine/threonine kinase activity typical of the NEK family as well as tyrosine kinase activity that is not a feature of other NEKs [10]. NEK1 has been implicated in ciliogenesis [11] and DNA damage response [12–15]. Additionally, NEK1 is highly expressed in mouse germ cells [16], where it appears to play essential roles in meiosis I and possibly also at later stages [17,18].

The Kat2J allele of NEK1 is a spontaneous point mutation within the coding sequence of Nek1 that results in a premature stop codon leading to a truncated protein lacking the both kinase domains [16]. Nek1Kat2J/kat2J mice display polycystic kidneys, dwarfism, anemia and male sterility [16,19]. Interestingly, while initial reports mention that Nek1Kat2J/kat2J females had low fertility rates [16,19], in our animal facility females no pregnancies are observed [17] possibly reflecting subtle background effects among mouse strains. Our previous studies of meiosis in Nek1Kat2J/kat2J mice showed that the loss of NEK1 activity induces aberrant retention of cohesin subunit Structural Maintenance of Chromosomes protein 3 (SMC3), at the end of meiotic prophase I [17]. More recently, we showed NEK1 regulates cohesin removal, in part, through regulation of wings apart-like homolog (WAPL) during meiotic prophase I [18], as part of the so-called Prophase pathway for cohesin removal. This regulation of WAPL by NEK1 is mediated through WAPL interactions with the cohesin-associated protein, PDS5 homolog B (PDS5B), and protein phosphatase 1 gamma (PP1γ), both of which also interact with NEK1 [18].

In the current study, and given the conserved function of NEK proteins in spindle assembly dynamics, we investigated the role of NEK1 downstream of prophase I events. Our results demonstrate that loss of NEK1 induces failure of meiotic spindle organization in both males and females, leading to elongated spindles, multipolar spindles, and abnormal chromosome congression. Concurrent with this, we observe altered levels and localization of two proteins known to regulate mitotic spindle dynamics: the unconventional myosin, myosin X (MYO10), and α-adducin (ADD1). Interaction of ADD1 with MYO10 is dependent on phosphorylation of ADD1 which allows for the interaction of these two proteins on mitotic spindles [20].
Depletion of ADD1 or changes in its phosphorylation status results in abnormal mitotic spindles [20]. Our studies demonstrate that loss of NEK1 kinase causes changes in MYO10 and ADD1 protein levels, and hyperphosphorylation and deamination of ADD1 caused at least in part by the abnormal function of PP1 during meiotic spindle formation. Interestingly spindle phenotypes seem not directly related to the role of NEK1 in prophase I events.

**Material and methods**

**Mice and genotyping**

All mouse studies were conducted with the prior approval of the Cornell Institutional Animal Care and Use Committee (Protocol 2010–0054). The mice were bred under pathogen-free, controlled temperature (22±1°C) and regulated humidity (50–55%) conditions with periods of light/dark of 12 h and food available ad libitum. Mice were euthanized using CO2 administration method.

The Nek1kat2j/kat2j line was obtained originally from Jackson Laboratory (Bar Harbor, Maine), and has been established in our mouse colony for more than nine years. Genotyping of this mouse line was performed following the protocol described elsewhere [17]. For the purposes of these studies, male mice were 8 weeks old and female where used at 24–28 days old. Homozygous mutant animals of (Nek1kat2J/kat2J) were compared with wild type (Nek1+/+) littermates on a C57Bl/6J background.

**Male and female spindle preparation**

Male spindle analysis was performed in testis from 8 week old mice following the protocol described elsewhere [21,22]. Briefly, following euthanasia, the testes were removed, decapsulated and placed in PBS. Using fine forceps, tubules were separated and analysed under a stereomicroscope. Differential light absorption of the tubules creates different zones that can be defined by different cell populations [22]. The differentent zones are consequence of the paracrine regulaiton by Sertoli cells, spermatogenesis proceeds in synchronized waves along the seminiferus tubules, and every given cross-section contains only certain cell types, that produces a different light absorption creating zones [22]. These zones are the weak spot that correspond to the spermatogenic stages XII-I, the strong spot (stages II-VI), the dark zone (stages VII-VIII) and the pale zone (stage IX-XI) We selected the zone corresponding to the spermatogenetic development XI and XII that are rich in meiotic cells during both meiosis I and meiosis II. Tubules were placed in fresh PBS and these required zones were excised and placed on poly-lysine-coated slides (P4981, Thermo Fisher). Tubule sections were fixed for 10 minutes (1% paraformaldehyde and 0.15% Triton-X, pH 9.2) and then squashed under a coverslip. Slides with the squashed tubules were put into liquid nitrogen for 20 seconds and thereafter the coverslip removed and washed with PBS-0.4% Kodak Photo flo (Kodak) (3 times for 5 minutes each). Slides were kept at -80˚C or stained immediately.

Female spindle analysis was performed with oocytes from 24–28 day old mice following our previously published protocol [23]. Briefly, after dissection, ovaries were collected in collection medium (Waymouth’s medium (11220035, Gibco), 10% Fetal bovine serum (26140079, Gibco), 1% penicillin-streptomycin (P4333-100ML, Sigma-Aldrich) and 0.1% sodium pyruvate (11360070, Gibco)). Oocytes were released from ovarian follicles using 30-gauge needles, and oocytes with germinal vesicles placed in EmbryoMax KSOM medium (MR-121-D, EMD Millipore). Oocytes were cultured for 6hr in EmbryoMax KSOM medium drops at 37˚C, 5% CO2. After incubation, oocytes were placed in fibrinogen-thrombin clot, incubated for 60 seconds at 37˚C, and rinsed with 1XPBS containing 2% Triton-X (BP151-500, Fisher Scientific) for 3 min. Oocytes were fixed for 30 minutes at 37˚C. Fixation was
followed by a 15 minute wash in 0.1% NGS (5 ml 10X PBS, 50 μL goat serum and 50 ml Milli-Q water). Oocytes were stored at 4˚C or stained immediately.

**Spindle immunofluorescence staining**

Male and female fixed spindles slides were washed in PBS containing 0.4% Kodak Photo flo (Kodak) for 5 min, followed by 0.1% PBS-Triton X and blocked in 1XPBS-antibody dilution buffer (ADB) before being incubated over night at 4˚C with the primary antibody. Primary antibodies used were: rabbit anti-ADD1 (GTX101600, Genetex. Dilution 1:100), rabbit anti-MYO10 (24565-1-AP, Proteintech. Dilution 1:100) and rabbit anti-β-tubulin (T8328, Sigma. Dilution 1:500). After overnight incubation, slides were washed to remove the unbound antibodies and incubate for 2 hours at 37˚C with Alexafluor™ secondary antibodies (Molecular Probes Eugene OR, USA). Slides were washed and mounted with Prolong Gold antifade (Molecular Probes). Image acquisition was performed using a Zeiss Imager Z1 microscope under 20X, 40X or 63X magnifying objectives, at room temperature. Images were processed using ZEN 2 (Carl Zeiss).

**Mass spectrometry**

Mass spectrometry was performed in the Cornell University Proteomics and Mass Spectrometry facility. 2D LC-MS/MS raw data files were acquired using Orbitrap Elite (Thermo Scientific). We performed a database search using Mascot searching against the SwissProt mouse database from Uniprot website (http://www.uniprot.org) using Mascot software version 2.3.02 (Matrix Science, UK). The default Mascot search settings were as follows: one missed cleavage site by trypsin allowed with fixed MMTS modification of cysteine, fixed four-plex iTRAQ modifications on Lys and N-terminal amines and variable modifications of methionine oxidation, deamidation of Asn and Gln residues, and 4-plex iTRAQ on Tyr for iTRAQ 4-plex analysis. One or two-missed cleavage site by trypsin allowed with fixed carboxamidomethyl modification of cysteine, fixed six-plex TMT modifications on Lys and N-terminal amines and variable modifications of methionine oxidation, deamidation of Asn and Gln residues, and 6-plex TMT on Tyr for TMT 6-plex analysis. The quantitative protein ratios were weighted and normalized by the median ratio with outlier removal set automatic in Mascot for each set of experiments. Only those proteins with ratios equivalents to two-fold increase or two-fold reduction were considered significant. To obtain the specific post-translational modification of the peptides we perfumed TiO2 enrichment followed by the proteomics analysis as was described above.

**Western blotting**

Whole testis protein and oocyte protein were extracted by sonication in RIPA buffer. Samples were boiled for 5 min in sample buffer, electrophoresed on SDS-polyacrylamide gels (8%) and transferred to nitrocellulose membranes. Primary antibody incubation was performed overnight at 4˚C at 1:1000 dilution (antibodies are the same used in spindle staining). Incubation with secondary antibodies was performed for two hours at room temperature (secondary HRP conjugated antibodies were obtained from Pierce, Life Technologies). Signal-detection was carried out using the superSignal substrate (Thermo Scientific). Loading control was performed using GAPDH-HPR (PA1-987-HRP, from ThermoFisher). Images were captured with BIO RAD Image Lab 5.1and analyzed by ImageJ version 1.49v (http://rsbweb.nih.gov/ij).
Results

Loss of NEK1 induces abnormal meiotic spindle formation and failed chromosome congression at the first meiotic division in males and females

We analyzed the role of NEK1 in spindle formation and chromosome orientation at MI in oocytes and spermatocytes from Nek1<sup>+/+</sup> and Nek1<sup>kat2j/kat2j</sup> mice. In male mice, we evaluated the shape, number of poles and misaligned chromosomes in both Nek1<sup>+/+</sup> (n = 100) and Nek1<sup>kat2j/kat2j</sup> (n = 100) spermatocytes. MI spindles from Nek1<sup>+/+</sup> spermatocytes showed a bipolar structure with the chromosomes aligned at metaphase plate (Fig 1a), while spindles from Nek1<sup>kat2j/kat2j</sup> spermatocytes showed defects in the structure of the spindle as well as in chromosome congression. These defects included MI spindles without a pole, with only one pole, with misaligned chromosomes (Fig 1b), and with multiple poles (Fig 1c). A total of 55% of spindles from Nek1<sup>kat2j/kat2j</sup> males were abnormal, while only 18% of the spindles from Nek1<sup>+/+</sup> male spermatocytes showed abnormalities (Fig 1d). Quantification of spindle abnormalities in Nek1<sup>+/+</sup> and Nek1<sup>kat2j/kat2j</sup> spermatocytes reveals that the most common abnormality is misaligned chromosomes. The percentage of spindles with misaligned chromosomes in Nek1<sup>+/+</sup> was 18%, while in Nek1<sup>kat2j/kat2j</sup> mice the percentage reached 38%. Other observed spindle abnormalities were absence of poles, one pole and multiple poles (Fig 1e).

The analysis of MI spindles in oocytes from Nek1<sup>+/+</sup> females showed a bipolar structure of the spindle and appropriate congression of the chromosomes at the metaphase plate (Fig 2a). However, MI spindles from Nek1<sup>kat2j/kat2j</sup> females showed a distortion of the structure of the spindle characterized by the presence of multiple spindles, mini spindles and misaligned chromosomes (Fig 2b, 2c and 2d) (for a complete analysis of the oocyte spindle dynamics, see S1 Fig). We evaluated the percentage of abnormal spindles in both wildtype oocytes (n = 100) and mutant oocytes (n = 100). In Nek1<sup>kat2j/kat2j</sup> female spindles, 82% of the MI spindles were abnormal, while only 22% of the Nek1<sup>+/+</sup> oocytes showed any abnormality (Fig 2e). Analysis of oocyte spindle defects showed that the most frequent defect is misaligned chromosomes: the number of oocytes with this defect 3.5 times higher in Nek1<sup>kat2j/kat2j</sup> (70 cells) compared to Nek1<sup>+/+</sup> oocytes (22 cells). No minispindles or multipole spindles were observed in Nek1<sup>+/+</sup> oocytes. Besides the differences observed in the spindle phenotype in male and female, our results indicate that NEK1 is required for spindle formation during meiosis.

Nek1<sup>kat2j/kat2j</sup> spermatocytes and oocytes have an abnormal spindle length and width

The appropriate length and width of the spindle are critical for establishing tension during segregation, and thus both parameters were evaluated them in male and female spindles. To perform this analysis in both male and female spindles we excluded all the cells that showed spindles without a pole, one pole, multipole, and minispindles, only those with two poles were evaluated. We measured the distance between poles in MI spermatocyte spindles and we observed a significant increase of the length of Nek1<sup>kat2j/kat2j</sup> males (27.9 μm ± 7.0 s.d.) compared to that of Nek1<sup>+/+</sup> males (23.1 μm ± 2.0 s.d.; Unpaired t test p = 0.0020; Fig 3a). Increases in MI spindle length in male spermatocytes were also accompanied with increases in the width in MI spindles from Nek1<sup>kat2j/kat2j</sup> male mice (26.7 μm, ± 6.4 s.d.) compared to that of Nek1<sup>+/+</sup> male mice (22.9 μm ± 2.1 s.d.; Unpaired t test p = 0.0097; Fig 3b). We also analyzed the length and width of the female spindles. Nek1<sup>kat2j/kat2j</sup> oocytes showed an increase in the length of the spindle in MI oocytes (31.6 μm ± 7.3 s.d.) compared to that found in Nek1<sup>+/+</sup> females (26.0 μm ± 2.3 s.d.; Unpaired t test p = 0.0007; Fig 3c). The lack of NEK1 also resulted in
Alteration in the width of the MI spindles in female meiosis. NeK1kat2j/kat2j oocytes showed a decrease in the width of the spindle (15.1 \mu m \pm 4.0 s.d.) compared to the NeK1+/+ oocyte spindles (17.0 \mu m \pm 1.7 s.d.; Unpaired t-test \( p = 0.039 \); Fig 3d). Taken together our results show that NEK1 is required for the establishment of the correct spindle length and width during meiosis in both male and female gametes.

Fig 1. Loss of NEK1 results in disrupted spindle morphology and chromosome congression during meiosis I in spermatocytes. (a-c) Immunofluorescence (IF) against \( \beta \)-tubulin (green) and DNA staining with DAPI (magenta) showing meiotic spindle morphology (panels a1-c1), with graphic representations below (panels a2-c2). (A) NeK1+/+ spermatocyte shows normal spindle morphology and chromosome congression at the midplate of the spindle; (b, c) Examples of aberrant spindle morphology in spermatocytes from NeK1kat2j/kat2j mice showing monopolar spindles with mislocalized chromosomes resulting from failed chromosome congression (arrowheads) in b and multipolar spindles in c (arrowheads). (d) Quantitation of abnormal spindles in spermatocytes from NeK1+/+ and NeK1kat2j/kat2j mice (n = 100 cells counted for each). (e) Quantitation cells with abnormal spindles by type of defect. Values are percentages \pm Standard deviation. * Indicates statistically significant differences (Unpaired t-test \( p < 0.05 \)).

https://doi.org/10.1371/journal.pone.0185780.g001
Lack of NEK1 results in deregulation of myosin X (MYO10) and α-adducin (ADD1)

Given the disrupted spindle apparatus during meiosis I, we investigated possible protein targets of NEK1 to explain this phenotype. In a previous study, we had performed Mass spectrometry (MS) screening using testis protein extracts from Nek1+/+ and Nek1kat2j/kat2j mouse mice [18]. Review of these data using GO terms for centrosome and centromeric proteins revealed no significant differences in these proteins levels in Nek1kat2j/kat2j mouse extracts compared to wildtype mice (S2 Fig). Several proteins associated with spindle formation and regulation were

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Fig 2. Loss of NEK1 results in disrupted spindle morphology and chromosome congression during meiosis I in oocytes. (a-d) Immunofluorescence (IF) against β-tubulin (green) and DNA staining with DAPI (magenta) showing meiotic spindle morphology (panels a1-d1), with graphic representations below (panels a2-d2). (a) Nek1+/+ oocyte shows normal spindle morphology and chromosome congression at the midplate of the spindle; (b-d) Examples of aberrant spindle morphology in oocytes from Nek1kat2j/kat2j mice showing multipolar spindles with or without errors in chromosome congression (arrowheads indicate three extra spindles formed in the oocyte in b and two additional spindles in c). (e) Quantitation of abnormal spindles in oocytes from Nek1+/+ and Nek1kat2j/kat2j mice (n = 100 cells counted for each). (f) Quantitation of cells with abnormal spindles by type of defect. Values are percentages ± Standard deviation. * Indicates statistically significant differences (Unpaired t-test p < 0.05).

https://doi.org/10.1371/journal.pone.0185780.g002

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Fig 3. Lack of NEK1 results in abnormal meiosis I spindle morphology in spermatocytes and oocytes. (a) Immunofluorescence (IF) against β-tubulin (green) and DNA staining with DAPI (magenta) showing a wildtype (top) and Nek1kat2j/kat2j (bottom) spermatocytes, along with measurements of spindle length (b) and width (c). Black bars are means (μm) ± Standard deviation. (d) Immunofluorescence (IF) against β-tubulin (green) and DNA staining with DAPI (magenta) showing a wildtype (top) and Nek1kat2j/kat2j (bottom) oocytes, along with measurements of spindle length (b) and width (c). Black bars are means (μm) ± Standard deviation. * Indicates statistically significant differences (Unpaired t-test p < 0.05).

https://doi.org/10.1371/journal.pone.0185780.g003
identified and quantified, however no differences between \(Nek1^{+/+}\) and \(Nek1^{kat2/kat2}\) mouse were observed for tubulins, actins and SAC proteins (S3–S5 Figs).

Previous reports showed that MYO10 has a direct function in spindle formation [24–26]. In contrast to the tubulins, actins, and other spindle proteins that did not appear to be altered in the absence of NEK1, we observed that the levels of the myosins, MYOS, MYO7A, MYO10 and MYO15, were significantly increased in testis protein extracts from \(Nek1^{1/2}\) mutant mice compared to wildtype littermates (S1 Table). By contrast, the myosins, MYO1B, MYO1C, MYO1D, MYO7A and MYO9B were unaltered. The function of MYO10 in spindle formation and integrity is related to its binding partner \(\alpha\)-adducin (ADD1)[20]. Our MS results revealed that ADD1 showed lower protein levels in \(Nek1^{kat2/kat2}\) male mice compared to wildtype male mice (S1 Table). The changes in protein levels observed by MS indicates that the loss of NEK1 results in an imbalance in the ratio MYO10/ADD1, and this could be affecting the proper spindle formation and function.

\(Nek1^{kat2/kat2}\) spermatocytes and oocytes show aberrant localization of myosin X (MYO10) on meiotic spindles

We evaluated the localization of MYO10 on MI spindles from male spermatocytes and female oocytes. Immunofluorescence (IF) analysis in \(Nek1^{+/+}\) spindles revealed that MYO10 localizes along the \(\beta\)-tubulin fibers specifically in mouse spermatocytes (Fig 4a). We analyzed the number of MYO10 foci per nucleus in the coronal plane (Fig 4b) and found an increase in the number of foci in the \(Nek1^{kat2/kat2}\) mice (75.4 ± 6.7 s.d.) compared to \(Nek1^{+/+}\) animals (66.3 ± 5.5 s.d.; Unpaired t test \(p = 0.0001\)). Analysis of transverse sections of male spindles revealed that there is a subtle accumulation of MYO10 at the spindle pole in \(Nek1^{+/+}\) male mice (Fig 4a), and this was disrupted in \(Nek1^{kat2/kat2}\) males (Fig 4b). In the transverse plane, we observed an increase in the MYO10 foci number in \(Nek1^{kat2/kat2}\) males (74.7 ± 5.4 s.d.) compared to the \(Nek1^{+/+}\) mice (61.7 ± 4.4 s.d.; Unpaired t test \(p = 0.0001\)). The increase in MYO10 focus counts was supported by our mass spectrometry analysis showing increased MYO10 protein in testis extracts from \(Nek1^{kat2/kat2}\) males compared to \(Nek1^{+/+}\) mice (S1 Table).

We also evaluated the MYO10 foci in \(Nek1^{+/+}\) oocytes (Fig 4c), where MYO10 localizes on tubulin fibers as foci during MI (39.7 ± 5.1 s.d.). In contrast to the situation in males, however, oocytes obtained from \(Nek1^{kat2/kat2}\) females showed more small and dispersed foci that do not co-localize with \(\beta\)-tubulin. However, counts of MYO10 foci that co-localized with \(\beta\)-tubulin were significantly reduced (23.3 ± 5.4 s.d.; Unpaired T test \(p = 0.0001\))(Fig 4d). This could indicate that the MYO10 is present but its capacity to bind to tubulin is reduced. Taken together, our results indicate that loss of NEK1 leads to mislocalization of MYO10 along meiotic spindles in both male and females germ cells, but that the regulation of MYO10 by NEK1 may be sexually dimorphic.

Localization of ADD1 on spermatocytes and oocytes is disrupted in absence of NEK1

Our previous MS results showed that ADD1 proteins levels are reduced in testis extracts from \(Nek1^{kat2/Kat2}\) mouse compared to wildtype littermates (S1 Table)[18]. Thus, we evaluated the localization and protein levels of ADD1 in both male and female spindles during meiosis I. ADD1 localizes both to the poles of the spermatocyte spindles (Fig 5a, asterisk) and with \(\beta\)-tubulin fibers of the spindle (Fig 5a, arrow heads). We evaluated the ADD1 focus number associated with the spindle structure in coronal and transverse planes. MI spindles from \(Nek1^{kat2/kat2}\) males showed a decreased ADD1 focus number (25.7 ± 7.8 s.d.) compared to MI spindles.
from Nek1+/+ males (40.3 ± 3.2 s.d.), along with mislocalization of ADD1 on the spindle poles on the coronal plane (Unpaired t test p = 0.001)(Fig 5b). Analysis of the transverse plane of spindles from Nek1kat2j/kat2j males showed a decreased focus number (32.6 ± 4.6 s.d.) compared to that of spindles from Nek1+/+ male (22.3 ± 5.5 s.d.; Unpaired t test p = 0.0001). Reduced ADD1 focus numbers were supported by reduced ADD1 protein levels analyzed by MS in testis extracts from NEK1 mutant mice and wildtype littermates (S1 Table). Thus, the effect of loss of NEK1 on ADD1 localization was the opposite to that seen for MYO10.

In oocytes from Nek1+/+ females, ADD1 also localized to the spindle poles as two large foci on each pole (3.6 ± 0.9 s.d.) (Fig 5c and 5d) (ADD1 signal along the tubulin fibers was observed but not quantified). This localization pattern was disrupted in Nek1 mutant females, with elevated levels of ADD1 foci observed in the absence of NEK1. This was associated with the appearance of multiple foci on the poles (6.08 ± 1.4 s.d.; Unpaired t test p = 0.0002), rather
than the two distinct large aggregations of ADD1. Again, as with the males, the change in ADD1 focus frequency on the meiotic spindles was the opposite to that seen for MYO10 (ADD1 foci increased on oocyte spindles in the *Nek1* mutant animals, whereas MYO10 foci decreased on oocyte spindles in the absence of NEK1). Taken together, these results demonstrate that loss of NEK1 alters the profile and ratios of ADD1 and MYO10 protein on meiosis I spindles in males and females, but that the effect may be sexually dimorphic.

### Loss of NEK1 activity induces abnormal phosphorylation of ADD1

During mitotic spindle formation mammalian cell lines, ADD1 binds to the motor domain of MYO10 on the spindle. ADD1 and MYO10 interaction on mitotic spindles is negatively regulated by phosphorylation. Phosphorylation of ADD1 induces the loss of the interaction between both proteins and therefore the complex unloads from the mitotic spindle [20]. Phosphorylation of ADD1 results in abnormal mitotic spindle morphology (elongation, multipolar...
spindles and aberrant chromosome alignment) and loss of its interaction with MYO10 [20]. We evaluated the proteins levels of ADD1 in testis lysates and isolated oocytes. Analysis of protein levels from testis lysates revealed a decrease in the protein levels in extracts from mutant mice compared to wildtype testis (Fig 6a; Unpaired t test p = 0.0031). We observed a clear double band in the Nek1kat2j/kat2j lysates, however this double band is not that obvious in the lysates from Nek1+/+ testes. Decreased levels of ADD1 and the presence of a heavier band in WB analysis of Nek1kat2j/kat2j mouse compared to wildtype littermates suggested that the lack of NEK1 activity affects ADD1 phosphorylation. To test this hypothesis, we screened our previous phosphoproteomics data obtained from Nek1 mutant animals, specifically focusing on changes in ADD1. Loss of NEK1 results in a significant increase in the phosphorylation of ADD1, but not MYO10 (Fig 6b) [18]. Specifically, loss of NEK1 results in hyperphosphorylation of ADD1 on serine 465 (S465) (Fig 6c). Interestingly, ADD1 also showed an increase in deamidation of asparagine 462 (N462). Thus, both post-translational modifications could be acting as a trigger to induce the degradation of the protein.

The results described above show that the lack of NEK1 induces changes in spindle formation and protein localization in both spermatocytes and oocytes. However, the phenotype in both sexes is slightly different. We evaluated the relative protein levels of ADD1 in oocytes of both Nek1+/+ and Nek1kat2j/kat2j oocytes. WB analysis revealed a decrease in ADD1 protein levels in Nek1kat2j/kat2j oocytes compared to Nek1+/+ oocytes (Fig 6d; Unpaired t test p = 0.0003). As in male, GV oocytes from Nek1kat2j/kat2j mice showed a double band, however oocytes from Nek1+/+ also showed a double band. In both wildtype and mutant, the slower migrating band had a stronger signal indicating that probably in oocytes the basal status of ADD1 is its phosphorylated state, at least at this stage of development. To evaluate the changes in the ADD1 protein levels according to oocyte maturation, we performed oocyte culture followed by WB. Our results showed that after 6h in culture, Nek1+/+ oocytes showed the loss of the heavier band, possibly indicating a change in ADD1 phosphorylation. However in the Nek1kat2j/kat2j oocytes we observe that both bands almost disappear indicating that there is a change in the phosphorylation in the mutants but a faster degradation of the protein in the mutant oocytes. These results suggest that the phosphorylation levels of ADD1 in the mutant mice depends on active phosphorylation of the protein by a kinase that is mis-regulated in the absence of NEK1, or by loss of activity of a phosphatase that controls the phosphorylated status of ADD1. We previously reported the interaction of PP1γ with NEK1 and its function regulating the prophase pathway during meiosis [18]. To test if the changes of ADD1 phosphorylation similarly depends on PP1γ activity, we cultured Nek1+/+ oocytes for 6h in the presence of the PP1γ inhibitor, Calyculin (Fig 6e). The WB analysis of these cultures showed that the inhibition of PP1γ induces a statistically significant increase in ADD1 protein levels, predominantly of the slower migrating band, suggesting that the inhibition of PP1γ maintains the phosphorylation status of ADD1. Thus, these results suggest that the lack of NEK1 action on ADD1 phosphorylation status may be the result of changes to PP1γ activity on ADD1.

**Discussion**

In eukaryotes, the structure orchestrating chromosome alignment and segregation during cell division is the microtubular spindle [3]. To ensure correct spindle dynamics, the spindle assembly checkpoint (SAC) becomes activated in situations where tension is not appropriately established between the chromosomes and the spindle poles, and this will block the progression of the cell cycle to prevent aberrant segregation. In addition to the canonical SAC, other levels of control have been described, including MYO10 [24–26]. In oocytes and frog embryos, disruption of MYO10 negatively affects nuclear anchoring and meiotic spindle formation [26],
Fig 6. Loss of NEK1 during meiosis is associated with hyper-phosphorylation and reduced ADD1 protein in a PP1γ-dependent manner. (a) Quantitation of ADD1 protein levels in Nek1+/+ testis extracts relative to GAPDH control. (b) ADD1 and MYO10 protein levels, expressed as a ratio of Nek1kat2j/kat2j / Nek1+/+, as determined by mass spectrometry. (c) ADD1 phosphopeptide and deaminated peptide sequence determined by mass spectrometry to be higher in Nek1kat2j/kat2j testis extracts. (d) Quantitation by western blotting of ADD1 protein levels in oocytes at 0h and after 6h of culture. (e) Quantification of ADD1 protein levels in wildtype oocytes cultured for 6h in vehicle (ethanol) and the PP1 inhibitor Calyculin A (CLA) (at doses of 2 and 20 nM). All values are means ± Standard deviation. * Indicate statistically significant differences (Unpaired t test p < 0.05 and one-way ANOVA followed by Dunnett’s multiple comparisons test, p < 0.05, for cultures with inhibitor)

https://doi.org/10.1371/journal.pone.0185780.g006
while depletion of MYO10 in frog epithelial cells induces abnormal spindle movements, spindle elongation, multi-spindle and pole fragmentation [25,27]. MYO10 has an amino-terminal globular head domain that harbors acting binding and ATPase activities, giving to the protein the capacity to bind F-actin. The tail of MYO10 has the MyTH4 and FERM domains that give to the protein the ability to interact with microtubules [26,28]. Accordingly, recent studies have demonstrated that loss of Myo10 results in spindle dysfunction, while overexpression of only the MyTH4 domain alone affects the structure and shape of the spindle [24].

In the current study, we present evidence to suggest that loss of NEK1 induces increases in MYO10 protein levels in spermatocytes, and that this in turn may result in spindle defects. Our interpretation that overexpression of Myo10 leads to spindle defects is in line with the suggestion that over-expression of the MyTH4 domain of MYO10 results in competition with the wildtype protein, leading to the displacement of the latter from the spindle [28]. Furthermore, it has been suggested that over-expression of only the MyTH4 may disrupt the functional link between F-actin, microtubules and MYO10. In our data (S1 Table), we observed an increase in the MYO10 protein levels without changes in F-actin or microtubules that could be inducing an imbalance in the protein levels provoking a similar phenotype to the overexpression of the MyTH4 domain. However, we could not eliminate the possibility that the lack of NEK1 could induce changes in other proteins or on the structure of MYO10 that induces mislocalization and/or abnormal function of MYO10. However, more studies using a MYO10 conditional knockout or siRNA against MYO10 will help to answer these possibilities.

ADD1 is an actin-binding protein that is important for membrane stabilization [29] and for cell-cell adhesion [30,31]. There are 3 isoforms of ADD with similar domain structures that are formed by NH$_2$-terminal head domain, a neck domain and a C-terminal domain. The C-terminal domain is characterized by it high contain of myristoylated alanine-rich C kinase substrate (MARCKS), this MARCKS related domain is necessary for its interaction with F-actin, spectrin, and calmodulin [28,32–34]. Previous studies in somatic cells showed that loss of ADD1 leads to a failure in mitotic spindle formation characterized by distortion, elongation, multipolar spindles and abnormal chromosome alignment [20]. Here, we show that loss of NEK1 leads to a reduction in ADD1 protein levels in spermatocytes, abnormal ADD1 distribution on the meiotic spindle of spermatocytes, and consequent disruption of spindle formation and integrity. These phenotypes observed in meiotic cells are highly reminiscent of the phenotypes observed in somatic cells lacking Add1 [20].

ADD1 and MYO10 interact via the MyTH4 domain of MYO10, and this interaction is critical for the correct spindle formation and function in mitotic cells [20,25,26,28]. Interaction of ADD1 to MYO10 is dependent on phosphorylation whereby phosphorylation of ADD1 by cyclin-dependent kinase 1 (CDK1) enables ADD1 to bind MYO10 on mitotic spindle [20]. Depletion of ADD1 or changes in phosphorylation status results in abnormal mitotic spindles (elongation, multipolar spindles and aberrant chromosome alignment) [20]. In the absence of NEK1 we observe an increase in the phosphorylation of ADD1 at S465 and deamination of N462. Such modifications could induce premature loss of the ADD1-MYO10 interaction, first by altered phosphorylation of the protein and then via degradation of the protein as a consequence of the deamidation, similar to the phenotypes observed in somatic cells [20]. However, it is unclear what kinase is phosphorylating ADD1 during meiosis, since the increased phosphorylation in the absence of NEK1 suggests the up-regulation of a kinase that is itself regulated by NEK1 (either directly or indirectly). Clear candidates could be CDKs, however, our MS results did not show any significant change in the profile of CDK in the absence of NEK1, and specifically no differences in CDK1 indicating that increases in phosphorylation of ADD1 does not depend of CDK1 pathway. Alternatively, the increased phosphorylation of ADD1 could suggest the existence of a phosphatase that is inactivated by the loss of NEK1, allowing
the hyperphosphorylation of protein. We previously showed that in Nek1 mutants, there is a
down regulation and abnormal phosphorylation in whole testis lysate of PP1γ, and our results
of oocyte cultures in the presence of a PP1 inhibitor suggest that the increase in ADD1 phos-
phorylation could be mediated by a similar mechanism.

Our results showed the disruption of the spindle, however some inconsistencies between
male and female gametes were observed. In both cases is clear that the lack of NEK1 is affecting
ADD1 and MYO10, but the phenotype is slightly different. Thus, loss of NEK1 results in an
increase in MYO10 localization on male meiotic spindles and a decrease in MYO10 localiza-
tion on female meiotic spindles. Conversely, loss of NEK1 induces a decrease in ADD1 locali-
zation on male spindles, while in females the lack of NEK1 induces an increment in focus
number but smaller in size, suggesting a disruption in the protein loading or acceleration in
protein degradation. It is possible, therefore, that the increased focus count for ADD1 associ-
ated with the oocyte spindle reflects lower amounts of total protein per focus and/or fragmen-
tation of the structures with which ADD1 is associating. This is supported by the different
appearance of ADD1 foci in NEK1-deficient oocytes compared to wildtype oocytes. Another
important difference in Nek1+/+ and Nek1kat2j/kat2j oocytes compared to spermatocytes is the
presence of a double band of ADD1 in the WB analysis, suggesting that in wildtype oocytes
there is a basal phosphorylated state of ADD1. However the main difference between WT and
mutant is that in the wildtype after culture the heavier band disappear but the lighter band still
present while in the mutant both bands disappear indicating the instability of the protein in
the absence of NEK1. Further analysis of ADD1 dynamics during oocyte meiosis will require
proteomics analysis of thousands of mouse oocytes, in order to understand the distribution
and status of spindle regulatory proteins in the presence and absence of NEK1.

Taken together, our data indicate that effect of NEK1 is sexually dimorphic with respect to
spindle association of key spindle-related proteins, as is often the case in other aspects of mei-
otic regulation, including recombination, chromosome pairing and synapsis [6,35–37]. Our
results showed, how the lack of NEK1 induced changes in both male and female spindle how-
ever the variation in the female phenotype could be related to how the spindle is formed. In
males the spindle formation depend on the centrosomes. In mitotic cells, centrosome’s func-
tions are regulated by other proteins of the NEK family such as NEK2, NEK6, NEK7 and
NEK9 that in the absence of NEK1 could be modifying the interactions and function of tubu-
lins or myosins [38–40]. However, female spindle formation does not depend of centrosomes,
thus a potential compensatory function of other NEK’s during the spindle formation is not
present; inducing the differences observed in the female phenotype. The broad spectrum of
abnormal spindles in female Nek1kat2j/kat2j oocytes, also could be associated to the “leakiness”
of spindle check point in females [41]. Besides, differences observed in the phenotype, the lack
of NEK1 ends up in male and female infertility [16,17].

In summary, we propose that the loss of NEK1 activity induces abnormal spindle formation
through a mechanism mediated by the imbalance of MYO10 and ADD1. The decreases in
ADD1 and it abnormal localization on the meiotic spindle is mediated by an increase its phos-
phorylation and deamination that leads to loss of interaction of MYO10 with ADD1. Taken
together our results show the importance of NEK1 in the control of spindle formation during
male and female meiosis.

**Supporting information**

S1 Table. Myosin proteins quantitation by mass spectrometry.
(DOCX)
S1 Fig. Dynamics of spindle formation in Nek1kat2j/kat2j oocytes. (a-f) Immunofluorescence against β-tubulin (green) and DNA staining with DAPI (magenta) showing the spindle dynamics in Nek1kat2j/kat2j oocytes. a) Germinal vesicle (GV); b) Germinal vesicle breakdown (GVBD); c) Prometaphase I (ProI); d and e) Metaphase I (MI); f) Telophase I (TeI). Scale bar 20μm (EPS)

S2 Fig. Analysis of centromere and centrosomal protein ratio Nek1kat2j/kat2j/Nek1+/+ by massspectrometry in whole testis lysates. (EPS)

S3 Fig. Analysis of tubulins protein ratio Nek1kat2j/kat2j/Nek1+/+ by massspectrometry in whole testis lysates. (EPS)

S4 Fig. Analysis of actins protein ratio Nek1kat2j/kat2j/Nek1+/+ by massspectrometry in whole testis lysates. (EPS)

S5 Fig. Analysis of spindle assembly checkpoint protein ratio Nek1kat2j/kat2j/Nek1+/+ by massspectrometry in whole testis lysates. (EPS)

Acknowledgments
We are grateful to Peter Borst for assistance in animal care and genotyping. We thank Dr. Sheng Zhang from Cornell Proteomics and Mass Spectrometry Facility for his advice in development of mass spectrometry experiments.

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