Ca\textsuperscript{2+} Release by IP\textsubscript{3} Receptors Is Required to Orient the Mitotic Spindle

Graphical Abstract

Highlights

- IP\textsubscript{3} receptors are required for mitotic spindle orientation
- Only IP\textsubscript{3} receptors with a functional channel restore spindle orientation
- Ca\textsuperscript{2+} release through IP\textsubscript{3} receptors is required for spindle orientation

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In Brief

Lagos-Cabré et al. show that Ca\textsuperscript{2+} release by IP\textsubscript{3} receptors (IP\textsubscript{3}Rs) regulates spindle orientation during metaphase. Spindles misalign in cells without IP\textsubscript{3}Rs. Alignment is restored by expression of IP\textsubscript{3}Rs but not by IP\textsubscript{3}Rs that cannot release Ca\textsuperscript{2+}. Ca\textsuperscript{2+} release by IP\textsubscript{3}Rs regulates astral microtubules and thereby spindle alignment.

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**Ca^{2+} Release by IP_3 Receptors Is Required to Orient the Mitotic Spindle**

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**SUMMARY**

The mitotic spindle distributes chromosomes evenly to daughter cells during mitosis. The orientation of the spindle, guided by internal and external cues, determines the axis of cell division and thereby contributes to tissue morphogenesis. Progression through mitosis requires local Ca^{2+} signals at critical steps, and because store-operated Ca^{2+} entry is inhibited during mitosis, those signals probably require Ca^{2+} release through inositol 1,4,5-trisphosphate receptors (IP_{3}Rs). In cells without IP_{3}Rs, astral microtubules around the daughter centrosome are shorter than those at the mother centrosome, and the mitotic spindle fails to align with the substratum during metaphase. The misalignment is due to the spindle ineffectively detecting internal cues rather than a failure of cells to recognize the substratum. Expression of type 3 IP_{3}R is sufficient to rescue spindle alignment, but only if the IP_{3}R has a functional pore. We conclude that Ca^{2+} signals evoked by IP_{3}Rs are required to orient the mitotic spindle.

**INTRODUCTION**

During mitosis, centrosomes nucleate microtubules to form the mitotic spindle, which then distributes chromatids equally to daughter cells. The orientation of the spindle is important because it determines the plane of cell division and which of the daughter cells will receive the oldest (mother) centrosome (Bergstrahl et al., 2017; di Pietro et al., 2016). Some stem cells, for example, selectively inherit the mother centrosome (Pelletier and Yamashita, 2012). For symmetric cell divisions, spindle alignment ensures that cellular components are shared equally between daughter cells, whereas for asymmetric divisions, spindle orientation determines whether one or both cells remain attached to the basement membrane (Hehnly et al., 2015; Lagos-Cabré and Moreno, 2008). Hence, spindle orientation ensures effective mitosis; it determines cell fate and which cells remain stem cells (Pelletier and Yamashita, 2012). Aberrant spindle alignment is associated with defective morphogenesis (Hehnly et al., 2015; Seldin et al., 2016), delamination of epithelia (Nakajima et al., 2013), and cancer (Bergstralh et al., 2017; Seldin et al., 2016). Spindle positioning is achieved by interaction of astral microtubules emanating from the centrosomes with a protein complex anchored to the cell cortex. In vertebrates, this ternary complex includes nuclear mitotic apparatus (NuMA), LGN (for leucine-glycine-asparagine motifs), and the G-protein G_{z} (Bergstrahl et al., 2017). How cells regulate the interactions of astral microtubules with the cell cortex to ensure effective orientation of the mitotic spindle is not fully understood (di Pietro et al., 2016).

Ca^{2+} signals are associated with many key steps during mitosis, notably, at nuclear envelope breakdown, the transition from metaphase to anaphase and during cytokinesis (Humeau et al., 2018; Poenie et al., 1985, 1986; Whitaker and Patel, 1990; Wong et al., 2005). Ca^{2+} signals typically arise from a combination of Ca^{2+} release from intracellular stores, predominantly within the endoplasmic reticulum (ER), and Ca^{2+} entry across the plasma membrane. Store-operated Ca^{2+} entry, where loss of Ca^{2+} from the ER triggers opening of Ca^{2+} channels in the plasma membrane, is the most widely expressed Ca^{2+} entry pathway, but it is completely inhibited during mitosis (Smyth et al., 2009; Yu et al., 2019). Hence, mitotic Ca^{2+} signals are likely to be due entirely to release of Ca^{2+} from intracellular stores (Ciapa et al., 1994), most likely mediated by opening of inositol 1,4,5-trisphosphate receptors (IP_{3}Rs), which are ubiquitously expressed intracellular Ca^{2+} channels (Rossi and Taylor, 2018). Vertebrates express three closely related IP_{3}R subunits (IP_{3}R1–3) that are differentially expressed and differ in both their sensitivity to IP_{3} and in their modulation by other signals, but each assembles to form homo- or hetero-tetrameric channels that open after IP_{3} binding (Alzayady et al., 2016). It is noteworthy that IP_{3}Rs are phosphorylated by several mitosis-related kinases, including polo-like kinase 1 (PLK1), cyclin-dependent kinase 1 (CDK1), and extracellular signal-regulated kinases 1 and 2 (ERK1/2), each of which regulates responses to IP_{3} (Ito et al., 2008; Prole and Taylor, 2016; Sathanawongs et al., 2015). Furthermore, mitosis is accompanied by substantial subcellular redistribution of the ER and IP_{3}Rs (Mitsuyama and Sawai, 2001; Parry et al., 2005).

We show that in cells without IP_{3}Rs, the mitotic spindle fails to align properly. Although the NuMA crescent aligns appropriately in cells lacking IP_{3}Rs, the spindle fails to align with that internal cue and astral microtubules around the daughter centrosome are shorter than those emanating from the mother centrosome.
Figure 1. Loss of IP$_3$Rs Causes Misalignment of Mitotic Spindles

(A) Typical projections of confocal z stacks from cells stained for chromosomes (blue), α-tubulin (green), and γ-tubulin (red) show spindle angles (α) during metaphase for a WT and HEK-IP$_3$R-KO cell. Dashed lines show substratum. Scale bars: 10 μm.

(B) Spindle angles for WT and HEK-IP$_3$R-KO cells (individual values, means ± SD from five experiments). **p < 0.01, Student’s t test.

(C) Frequency distribution of spindle angles (n = 68–71 cells, from five experiments). ***p < 0.001, χ$^2$ test for trend.

(D) Typical western blots (WBs) for IP$_3$R subtypes in HEK cells treated with siRNAs to all three IP$_3$R subtypes or non-silencing (NS) siRNA.

(E) Summary results show IP$_3$R expression determined by quantification of WB for cells treated with IP$_3$R siRNA relative to NS siRNA (% means ± SD, n = 4).

(legend continued on next page)
Expression of IP$_3$R3 rescues spindle alignment, but only if the IP$_3$Rs have a functional pore. We conclude that Ca$^{2+}$ release by IP$_3$Rs, which accumulate around centrosomes during metaphase and more so at the mother centrosome, is required for spindle alignment.

RESULTS

IP$_3$ Receptors Are Required for the Mitotic Spindle to Align with the Substratum

Human embryonic kidney (HEK) cells without IP$_3$Rs (HEK-IP$_3$R-KO cells) and wild-type (WT) cells grew at similar rates (Figure S1A), confirming that IP$_3$Rs are not essential for proliferation (Ando et al., 2018; Atakpa et al., 2019; Sugawara et al., 1997). Time-lapse images of dividing cells showed that most WT cells formed a metaphase plate that aligned perpendicular to the substratum, allowing each daughter cell to remain attached to the substratum after cytokinesis (Figure S1B). This behavior is common to almost all epithelial cells and cell lines (Hehnly et al., 2015). However, many HEK-IP$_3$R-KO cells formed a metaphase plate that was not perpendicular to the substratum, allowing only one daughter cell to remain attached, whereas the other was expelled into the medium (Figures S1C and S1D; Video S1). Imaging of cells expressing mCherry-α-tubulin and histone-2B-EGFP, to identify microtubules and chromosomes, respectively, showed that mitotic spindles in WT cells remained parallel to the substratum throughout metaphase, whereas, in HEK-IP$_3$R-KO cells, the spindle rotated (Videos S2A and S2B). The misaligned spindles in HEK-IP$_3$R-KO cells were often associated with aberrant mitoses, including nuclear division without cytokinesis (Video S2C), consistent with many cells having several nuclei or multinuclear spindles (Figures S1E–S1H). The results are also consistent with a small interfering RNA (siRNA) screen, where loss of IP$_3$R1 was associated with abnormal cytokinesis (Kittler et al., 2004). These observations prompted us to examine the relationship between IP$_3$Rs and the mitotic spindle.

We measured spindle angles relative to the substratum for semi-confluent HEK cells during metaphase (Figure 1A). In WT cells, most spindles aligned parallel to the substratum (a perpendicular metaphase plate), with an average spindle angle of 15.5° ± 16.4° (mean ± SD, n = 71 cells). Because the angles always have positive values, any variation causes the mean to deviate from the 0° that would indicate perfect alignment. In HEK-IP$_3$R-KO cells, the bipolar spindles were more randomly oriented, evident from both the larger average spindle angle (31.6° ± 21.0°, n = 68) and the wider distribution of the angles (Figures 1B and 1C). Similar results were obtained using γ-tubulin to define spindle angles (Figure S1C) and from analyses of HAP1 cells with and without IP$_3$Rs (Figures S2A–S2C).

Because the cell lines lacking IP$_3$Rs were generated by selection after CRISPR/Cas9-mediated gene disruption (Alzayady et al., 2016; Atakpa et al., 2018), the misaligned spindles might have arisen through effects unrelated to the loss of IP$_3$Rs. We, therefore, used siRNAs directed to each IP$_3$R subtype to acutely reduce expression of all three IP$_3$R subtypes in HEK cells. The siRNA treatment reduced expression of the IP$_3$R subtypes similarly (Figures 1D and 1E). Spindle angles were significantly perturbed in siRNA-treated cells (26.9° ± 17.5°, n = 75), although less so than in HEK-IP$_3$R-KO cells (35.2° ± 21.2°, n = 64) (Figure 1F). The distribution of spindle angles confirmed that loss of IP$_3$Rs by siRNA or gene disruption had similar effects (Figure 1G).

HEK cells express all three IP$_3$R subtypes (Mataragka and Taylor, 2018). In cells expressing only a single IP$_3$R subtype, spindle angles were aberrant in cells expressing only IP$_3$R1 (23.4° ± 14.7°, n = 72), but more normal in cells expressing only IP$_3$R2 (18.1° ± 14.4°, n = 51) or IP$_3$R3 (17.7° ± 13.9°, n = 66) (Figures S2D–S2F). We cannot conclude from these results that IP$_3$R1 is incapable of contributing to spindle alignment because the HEK-IP$_3$R1 cells may have acquired other defects during selection, but the results do establish that the requirement for IP$_3$Rs can be satisfied by IP$_3$R2 or IP$_3$R3. Our subsequent studies focus on IP$_3$R3 because plasmids encoding it are more reliably propagated than those expressing IP$_3$R2, and there is a better antibody to IP$_3$R3.

In HEK-IP$_3$R-KO cells, the spindle angle (34.4° ± 17.4°, n = 53) was significantly rescued by expression of EGFP-IP$_3$R3 (22.9° ± 18.3°, n = 65) to values that were not significantly different from the mock-transfected WT cells (16.2° ± 13.4°, n = 55) (Figures 1H–1J). We confirmed that the EGFP tag did not affect IP$_3$R3 function by demonstrating that, for matched levels of IP$_3$R3 expression (and comparable to WT cells), rescue of spindle alignment was indistinguishable for EGFP-IP$_3$R3 (22.9° ± 18.3°) and untagged IP$_3$R3 (22.0° ± 16.4°, n = 63 cells) (Figures 1H–1J and S3). Furthermore, expression of IP$_3$R3 at levels substantially exceeding native levels did not perturb spindle alignment (Figure S3A). In WT cells, however, endogenous IP$_3$R3 expression inversely correlated with spindle angle, suggesting that native levels of IP$_3$R expression may be limiting for appropriate spindle alignment (Figure S3A). The results so far establish that IP$_3$Rs are required for the mitotic spindle to align.
with the substratum and that IP$_3$R3 (or EGFP-IP$_3$R3) is sufficient to meet that need.

**IP$_3$ Receptors Are Required to Align Spindles with the NuMA Complex**

Spindle angles were measured relative to the substratum (Figures 1A and S1B) using semi-confluent cells, in which most cells contact at least one neighbor. We therefore considered whether spindle misalignment in cells without IP$_3$Rs arose from ineffective decoding of appropriate intracellular signals by the spindle apparatus or from ineffective detection of external cues, for example β1-integrin-mediated interaction with the extracellular matrix (Iwano et al., 2015) or cadherin-mediated interactions between cells (Hart et al., 2017).

Using synchronized dividing cells stained with a lipid marker, we confirmed that HEK-IP$_3$R-KO cells adhere normally and expose similar areas of plasma membrane (PM) to the substratum as WT cells (Figures 2A and S4A). In sparsely distributed synchronized WT cells expressing mCherry-α-tubulin, spindles aligned with the substratum (17.6° ± 14.1°, n = 26) (Figures 2B and S4B), consistent with published results from isolated HeLa cells (Toyoshima et al., 2007), but again, the spindles were misaligned in HEK-IP$_3$R-KO cells (31.4° ± 20.3°, n = 30) (Figures 2B–2D). We conclude that
The oldest centrosome, more effectively nucleates microtubules, forms more extensive astral microtubules during mitosis, contributes to cell polarity, and can affect cell fate (Hehnly et al., 2015; Yamashita, 2009). In WT HEK cells and in HEK-IP3R-KO cells expressing EGFP-IP3R3 (wherein the distribution of IP3R3 mimics that of WT cells; Figure S5B), IP3R3 concentrated most around the mother centrosome during metaphase (Figures 3A and 3B). Indistinguishable results were obtained using immunostaining (WT cells) and EGFP-fluorescence (HEK-IP3R-KO cells expressing EGFP-IP3R3) (Figure 3B). These observations prompted us to determine separately the length of astral microtubules around mother and daughter centrosomes. The results indicate that in HEK-IP3R-KO cells, the astral microtubules around the daughter centrosome are shorter than those around the mother centrosome (Figures 3C, 3D, S5C, S5D, S5F, and S5G). There were no significant differences between cells with and without IP3Rs in the length of the mitotic spindle (9.44 ± 1.27 μm, n = 53 in WT cells, and 9.99 ± 1.41 μm, n = 57 in HEK-IP3R-KO cells, mean ± SD), the distance between the cortex and mother centrosome (5.36 ± 2.35 μm, n = 26 and 4.86 ± 2.85 μm, n = 25) or daughter centrosome (5.81 ± 2.57 μm and 4.55 ± 2.02 μm), the fraction of the plus ends of microtubules (identified with end-binding protein 3, EB3) abutting the cortex around the mother or daughter centrosomes (Figures S5C–S5E), or the density of EB3 puncta around the centrosomes (Figure S5H).

Collectively, these results establish that IP3Rs are required for centrosomes to align appropriately with internal cues provided by the nucleus.
by the NuMA complex and that astral microtubules radiating from mother and daughter centrosomes are differentially affected by the loss of IP$_3$Rs.

**Ca$_{2+}$ Release by IP$_3$ Receptors Is Required for Spindle Alignment**

IP$_3$Rs are best known for releasing Ca$^{2+}$ from the ER (Rossi and Taylor, 2018), but additional proteins associate with IP$_3$Rs (Prole and Taylor, 2016), and many IP$_3$Rs appear not to release Ca$^{2+}$ in intact cells (Thillaiappan et al., 2017). We, therefore, considered whether Ca$^{2+}$ release through IP$_3$Rs was required for spindle alignment. Mutation of a single residue within the pore of IP$_3$R1 prevents it from conducting Ca$^{2+}$ (Boehning et al., 2001; Dellis et al., 2008). We mutated the equivalent residue (D2477A) in EGFP-IP$_3$R3 (EGFP-IP$_3$R3D/A) and confirmed that it prevented IP$_3$ from evoking Ca$^{2+}$ release (Figures S4F–S4H). Expression of EGFP-IP$_3$R3 in HEK IP$_3$R-KO cells rescued spindle alignment, and the similar lengths of astral microtubules at mother and daughter centrosomes were restored, but comparable expression of EGFP-IP$_3$R3D/A rescued neither feature (Figures 4A–4C, S5F, and S5G). These results establish that Ca$^{2+}$ release through IP$_3$Rs is required for mitotic spindles to align properly (Figure 4D).

**DISCUSSION**

Progression through the cell cycle is controlled by regulated degradation of cyclins, but local Ca$^{2+}$ signals are also important at critical stages (Keith et al., 1985; Parry et al., 2005; Zhao et al., 2019), including nuclear envelope breakdown, the transition from metaphase to anaphase and cytokinesis. The mechanisms are largely unresolved, but because store-operated Ca$^{2+}$ entry is inhibited during meiosis and mitosis (Smyth and Putney, 2012; Yu et al., 2019), the Ca$^{2+}$ signals are probably evoked by IP$_3$Rs. We have shown that cells can proliferate without IP$_3$Rs (Figure S1A) (Alzayady et al., 2016; Ando et al., 2018; Atakpa et al., 2019; Sugawara et al., 1997), but their behavior is compromised. In some cells without IP$_3$Rs, the mitotic spindle is aberrant and cytokinesis sometimes fails (Figure S1). However, most cells divide...
successfully but have spindles that fail to align with the substra-
tum because they no longer associate appropriately with the
NuMA complex (Figures 1 and 2). The defects are reversed by
expression of IP₃R3, but only if it has a functional Ca²⁺ pore (Fig-
ure 4). This is consistent with recent work suggesting that local
Ca²⁺ signals occur near centrosomes throughout mitosis, and
abolishing them at one centrosome perturbs mitosis (Helassa
et al., 2019). It is noteworthy that variations in expression of
endogenous IP₃Rs are associated with the reliability of spindle
alignment: WT cells with fewer IP₃Rs are more likely to have mis-
aligned spindles (Figure S3A).

Astral microtubules link the NuMA complex to the pericentri-
olar material (PCM) that surrounds each centrosome (Figure S4C).
IP₃Rs concentrate most around the mother centrosome (Figures
3A and 3B), which nucleates astral microtubules that are more
abundant and longer than those associated with the daughter
centrosome (Hehnly et al., 2015; Yamashita, 2009). There is
a significant disparity in the length of astral microtubules
emanating from the mother and daughter centrosomes in cells
without IP₃Rs: astral microtubules at the mother pole are longer
than those at the daughter pole (Figures 3C, 3D, S5F, and S5G),
although they appear to make similar numbers of contacts with
the cortex at both poles in cells with or without IP₃Rs (Figures
S5C–S5E). Our results indicate an asymmetric relationship be-
tween IP₃Rs and the mother and daughter centrosomes. The
mother centrosome has greater capacity to nucleate microtu-
bules and associates with additional proteins (Huhn et al.,
2017) and IP₃Rs concentrate most around it (Figures 3A and
3B), and in cells without IP₃Rs, there is a relative lengthening
of astral microtubules around the mother centrosome (Figures
3C, 3D, SSF, and SSG). We speculate that the asymmetric
lengths of astral microtubules around mother and daughter cen-
trosomes in cells without IP₃Rs may generate uneven forces be-
tween them and the cortex (Howard and Garzon-Coral, 2017),
leading to spindle misalignment.

We conclude that Ca²⁺ release through IP₃Rs is required for
the mitotic spindle to align appropriately, probably through regu-
lation of astral microtubules by IP₃R-evoked Ca²⁺ signals (Fig-
ure 4D). Future experiments should assess whether the Ca²⁺ sig-
als that occur during metaphase (Helassa et al., 2019) regulate
microtubules and their motors or deliver Ca²⁺ locally to the mito-
chondria to allow local delivery of ATP (Zhao et al., 2019) and
thereby regulation of microtubule activity. Our results reveal
that IP₃R-evoked Ca²⁺ signals are an important means of regu-
lating the mitotic spindle.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.
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AUTHOR CONTRIBUTIONS

R.L.-C. performed most experiments. A.I. analyzed IP₃R localization. C.W.T.
supervised the study and wrote the manuscript. All authors contributed to
data analysis and reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Antibodies          |        |            |
| WB, western blot; IC, immunocytochemistry |        |            |
| Mouse monoclonal anti-a-tubulin (IC, 1:1000) | Sigma Aldrich, Gillingham, UK | cat# T6074; RRID: AB_477582 |
| Rabbit anti-1-tubulin (IC, 1:400) | GeneTex, CA, USA | cat# GTX113286; RRID: AB_1952442 |
| Rabbit anti-cenexin (aka ODF2) (IC, 1:200-400) | GeneTex | cat# GTX114594; RRID: AB_2814951 |
| Mouse monoclonal anti-b-actin (WB, 1:5000) | Cell Signaling Technology, Danvers, MA, USA | cat# 3700; RRID: AB_2242334 |
| Mouse monoclonal anti-IP3R1 (WB, 1:1000) | Abclonal, Tokyo, Japan. | cat# A7905; RRID: AB_2770019 |
| Rabbit anti-IP3R2 (WB, 1:1000) | Pocono Rabbit Farm & Laboratory, Inc., Canadensis, PA, USA | Custom-made for our laboratory (Mataragka and Taylor, 2018) |
| Mouse monoclonal anti-IP3R3 (WB, 1:1000; IC, 1:200) | BD Bioscience, NJ, USA | cat# 610313; RRID: AB_397705 |
| Rabbit anti-IP3R (recognizes all IP3R subtypes) (WB, 1:1000; Figure 1D) | Pocono Rabbit Farm & Laboratory, Inc. | Custom-made for our laboratory (Mataragka and Taylor, 2018) |
| Rabbit anti-IP3R (recognizes all subtypes) (WB, 1:1000; Figure S2D), Although described as anti-IP3R1, the antigenic peptide is common to all three IP3R subtypes. | Cell Signaling Technology | cat# 8568; RRID: AB_10890699 |
| Rat monoclonal anti-GFP (WB, 1:2000) | Chromotek, Munich, Germany | cat# 3h9-100; RRID: AB_10773374 |
| Rat monoclonal anti-EB3 antibody (IC, 1:100) | AbCam, Cambridge UK. | cat# Ab53360; RRID AB_880026 |
| IgG-HRP (WB, 1:5000) | Santa Cruz Biotechnology | cat# sc-516102; RRID: AB_2687626 |
| Mouse anti-rabbit IgG-HRP (WB 1:5000) | Santa Cruz Biotechnology | cat# sc-2357; RRID: AB_628497 |
| Goat anti-rat IgG-HRP (WB, 1:5000) | Santa Cruz Biotechnology | cat# sc-2006; RRID: AB_1125219 |
| Alexa Fluor 647 goat anti-rabbit IgG (IC, 1:400) | ThermoFisher | cat# A21244; RRID: AB_141663 |
| Alexa Fluor 488 goat anti-mouse IgG (IC, 1:400) | ThermoFisher | cat# A10667; RRID: AB_2534057 |
| Alexa Fluor 568 goat anti-mouse IgG (IC, 1:400) | ThermoFisher | cat# A11031; RRID: AB_144696 |
| Alexa Fluor 488 goat anti-rat IgG (IC, 1:100) | ThermoFisher | cat# A11006; RRID: AB_141373 |
| Alexa Fluor 568 goat anti-rat IgG (IC, 1:100) | ThermoFisher | cat# A11077; RRID: AB_141874 |
| Goat anti-rabbit IgG conjugated to CF405M (IC, 1:100) | Sigma | cat# SAB4600461 |
| GFP-Booster ATTO488 nanobody (IC, 1:500) | Chromotek | cat# gba488-100; RRID: AB_2631386 |

### Chemicals, Peptides, and Recombinant Proteins

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Bovine serum albumin (BSA) | Europa Bio-Products, Ely, UK | cat# EQBAH64 |
| Calbyte 590 AM | AAT Bioquest, Sunnyvale, CA, USA | cat# 20700 |
| Carbamoylcholine chloride (carbachol, CCh) | Sigma Aldrich | cat# C-4382 |
| cComplete, EDTA-free protease inhibitor cocktail | Roche | cat# 11836153001 |
| 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) | Sigma Aldrich | cat# D9542 |
| 3',3'-Diethyloxacarbocyanine iodide (DHCC) | Sigma Aldrich | cat# 318426 |
| Dulbecco’s Modified Eagle’s Medium (DMEM)/F-12 with GlutaMAX | ThermoFisher | cat# 31331093 |
| ECL Prime western blotting detection reagent | Amersham | cat# RPN2236 |
| Fibronectin (human) | Merck Millipore, Watford, UK | cat# FC010 |
| Fetal bovine serum (FBS) | Sigma Aldrich | cat# 094N3341 |
| Gibco TrypLE Express | ThermoFisher | cat# 12605010 |
| HEPES | Merck Millipore | cat# 391338 |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HiPerFect           | QIAGEN, Hilden, Germany | cat# 301705 |
| Ionomycin           | Cambridge Bioscience | cat# CAYM11932 |
| Iscove’s Modified Dulbecco’s Medium (IMDM) | ThermoFisher | cat# 12440-053 |
| NucBlue Live ReadyProbes reagent | ThermoFisher | cat# R37606 |
| PVDF iBlot transfer stack | ThermoFisher | cat# IB401031 |
| Run Blue (4-12%) SDS gel | Expedeon, San Diego, CA, USA | cat# NXG40812 |
| Thymidine           | Sigma Aldrich | cat# T9250 |
| TransIT-LT1 reagent | MirusBio, WI, USA | cat# MIR 2300 |
| Tris                | ThermoFisher | cat# BP152-1 |
| Tween-20            | Sigma-Aldrich | cat# T5927 |
| Trypan Blue         | ThermoFisher | cat# T10282 |
| Vectashield         | VectorLabs, Burlingame, CA, USA | cat# H-1000 |
| Wheat germ agglutinin (WGA)-CF405M | Biotium, CA, USA | cat# 29028-1 |
| Wheat germ agglutinin (WGA)-CF568 | Biotium | cat# 29077-1 |

### Critical Commercial Assays

| Critical Commercial Assays | SOURCE |
|---------------------------|--------|
| QuickChange Lightning site-directed mutagenesis kit | Agilent, Santa Clara, CA USA | cat# 210515 |

### Experimental Models: Cell Lines

| Experimental Models: Cell Lines | SOURCE |
|--------------------------------|--------|
| HEK cells (Dr D. Yule (University of Rochester, NY, USA)) | Dr D. Yule (University of Rochester, NY, USA) |
| HEK-IP₃R-KO cells (Kerafast, Boston, MA, USA) | cat# EUR030 |
| HEK-IP₃R1 cells (Kerafast) | cat# EUR031 |
| HEK-IP₃R2 cells (Kerafast) | cat# EUR032 |
| HEK-IP₃R3 cells (Kerafast) | cat# EUR033 |
| HAP1 cells (Horizon Discovery, Cambridge, UK) | cat# C631 |
| HAP1-IP₃R-KO cells (Atakpa et al., 2018) | Available from Horizon Discovery |

### Oligonucleotides

| Oligonucleotides | SOURCE | IDENTIFIER |
|------------------|--------|------------|
| IP₃R1 siRNA      | QIAGEN | cat# Hs_ITPR1_4 FlexiTube siRNA (SI00034545) |
| IP₃R2 siRNA      | QIAGEN | cat# Hs_ITPR2_1 FlexiTube siRNA (SI00034552) |
| IP₃R3 siRNA      | QIAGEN | cat# Hs_ITPR3_1 FlexiTube siRNA (SI00034580) |
| Non-silencing (NS) siRNA | QIAGEN | cat# 1027281 |

### Recombinant DNA

| Recombinant DNA | SOURCE | IDENTIFIER |
|-----------------|--------|------------|
| pcDNA3.2/DEST-EGFP-IP₃R3 (rat) | (Pantazaka and Taylor, 2011) | N/A |
| pcDNA3.2/V5DEST-IP₃R3 (rat) | (Tovey et al., 2010) | N/A |
| pShuttle mCherry-α-tubulin | Addgene | Addgene plasmid # 26768; RRID: Addgene_26768 (Matov et al., 2010) |
| Histone 2B-EGFP | Addgene | Addgene plasmid # 11680; RRID: Addgene_11680 (Kanda et al., 1998) |
| GFP-ER | (Wozniak et al., 2009) | N/A |

### Software and Algorithms

| Software and Algorithms | SOURCE |
|-------------------------|--------|
| GraphPad Prism 6.0      | GraphPad Software, 6.0 | [https://www.graphpad.com/](https://www.graphpad.com/) |
| Excel                   | Microsoft, 2007 | N/A |
| FIJI                    | [https://fiji.sc/](https://fiji.sc/) |
| GeneTools, version 4    | Syngene, Cambridge, UK | [https://www.syngene.com](https://www.syngene.com) |
| MetaMorph Microscopy Automation and Image Analysis | Molecular Devices, San Jose, CA | [https://www.moleculardevices.com](https://www.moleculardevices.com) |
and BSA (2%). Cells were incubated with primary antibody (1 hr, 20°C) in Tris-HCl, 150 mM NaCl, pH 7.5) with Tween-20 (0.1%) and Triton X-100 (0.1%), and then blocked in TBS containing Tween (0.1%).

Cell Reports

EXPERIMENTAL MODEL AND SUBJECT DETAILS

We used two cell lines, human embryonic kidney (HEK) 293 cells and HAP1 cells (near-haploid) and their derivatives. At the time of the study, these were the only mammalian cell lines in which all three IP3R subtypes had been disrupted. HEK293 cells are hypotriploid (CML) cell line, KBM-7. The cell line in which genes encoding all three IP3R subtypes were disrupted by CRISPR/Cas9 (HAP1 IP3R-KO) has been described previously (Atakpa et al., 2018). We have not independently verified the authenticity of the cell lines.

METHOD DETAILS

Immunochemistry

Cells (10^5/well) grown (24 hr) on 16-mm round glass coverslips (N° 0, VWR, International, Radnor, PA, USA) or 35-mm glass-bottomed imaging dishes (#P35G-1.0-14-C, MatTek Corporation, Ashland, MA, USA) coated with human fibronectin (50 μg/mL). Transient transfection with pcDNA3.2/V5DEST-IP3R3, pcDNA3.2/DEST-EGFP-IP3R3, pcDNA3.2/DEST-EGFP-IP3R3D/A, pShuttle mCherry-α-tubulin or H2B-EGFP plasmids used TransIT-LT1 reagent (1 μg DNA/3 μL reagent) according to the manufacturer’s instructions. Transfection with siRNA against each IP3R subtype (40 nM of each) or a non-silencing (NS) siRNA (120 nM) used HiPerfect transfection reagent according to the manufacturer’s instructions. Cells were used after 48 hr. pcDNA3.2/DEST-EGFP-IP3R3 was used to modify the coding sequence of EGFP-IP3R3 from D2477 (CGA) to A (GCC) using primers 5’-TGCGGAGGATGGCGCC-3’ and 5’-CGGCGTGCGGCCCATCTCCGGCGA-3’ and the QuickChange Lightning site-directed mutagenesis kit according to the manufacturer’s instructions. Sequencing of the entire coding sequence confirmed the single mutation.

Most experiments used non-synchronized cells, but to obtain sufficient cells in a field for analyses of isolated cells, cells were synchronized using either a single- or double-thymidine block. Thymidine arrests cells at the G1/S boundary by inhibiting DNA synthesis, and cells then synchronously enter S phase when thymidine is removed. For the single-block, HEK cells (10^5/well) grown on 35-mm imaging dishes coated with human fibronectin (50 μg/mL) were incubated with thymidine (2 mM) for 18-24 hr, washed three times in phosphate-buffered saline (PBS) and incubated in fresh medium for 7-10 hr before analysis. For the double-block, cells were incubated with thymidine (2 mM, 18 hr), washed, incubated in thymidine-free medium (9 hr), incubated again with thymidine (2 mM, 18 hr), washed and incubated in fresh medium (8-10 hr). We used these methods to avoid synchronization protocols that rely on perturbation of microtubules.

A Countess automated cell counter (ThermoFisher) was used to count cells in medium containing 0.2% Trypan Blue.
used for TIRFM and wide-field imaging. For quantification of IP<sub>3</sub>R3 immunostaining (Figures S3A and S3B), we used the background-corrected complete z stack from confocal images.

**Live-Cell Imaging**

Cells transfected to express mCherry-α-tubulin alone or with H2B-EGFP, and grown in imaging dishes were washed and incubated in HEPES-buffered saline (HBS: 135 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl<sub>2</sub>, 1.5 mM CaCl<sub>2</sub>, 11.5 mM glucose, 11.6 mM HEPES, pH 7.4) containing FBS (10%). Wide-field images of cells in HBS were collected at 1-min intervals using an Olympus IX83 microscope with a 100x objective (NA, 1.49) within an enclosed cabinet (37°C, 5% CO<sub>2</sub>) using 488/525 nm (excitation laser/emission filter) and 561/630 nm lasers, and an iLas2 targeted laser illumination system.

**Measurement of Contact Areas Between Cells and Substratum**

Synchronized HEK cells were incubated in HBS with 3,3'-dihexyloxacarbocyanine iodide (DHCC, 20 μg/mL, ~1 min). Live cells were imaged within 20 min by TIRFM and confocal microscopy (488/525 nm) using an Olympus IX83 microscope with 100x objective (NA, 1.49) to determine the areas contacting the substratum and across a mid-section of each cell.

**Measurements of Cytosolic Ca<sup>2+</sup> Signals**

HEK cells in HBS were incubated with Calbryte 590-AM (5 μM, 1 hr, 20°C in darkness), washed and incubated in HBS (45 min, 20°C) before imaging. Wide-field fluorescence images (488/525 nm to detect EGFP, 561/630 nm to detect Calbryte 590) were collected at 500-ms intervals using an Olympus IX83 microscope with a 100x objective (NA, 1.49). After subtraction of background fluorescence (from an area outside the cell), changes in fluorescence (F/F<sub>0</sub>) are reported relative to basal fluorescence (F<sub>0</sub>).

**Western Blots**

Cells were scraped into cold RIPA medium (1 mM Tris HCl, 15 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100, pH 7.5) containing protease inhibitors (cOmplete, EDTA-free protease inhibitor cocktail) and lysed using a syringe and 18G needle. The protein content of the supernatant (10,000 x g, 10 min) was quantified using a Bradford assay with BSA as standard. Proteins were separated using 4%–12% Run Blue Bis-tris gels, transferred to a polyvinyl difluoride (PVDF) membrane using an iBlot gel-transfer system, blocked (1 hr, 20°C) in TBS with Tween-20 (0.1%) and BSA (5%), and incubated (1 hr, 20°C) with primary antibody in fresh blocking buffer. After three washes, the membrane was incubated with HRP-conjugated secondary antibody (1 hr, 20°C) and washed three times. Bands were visualized with ECL Prime western blotting detection reagent using a GeneTools Syngene PXi chemiluminescence detection system, and quantified using FIJI (after subtracting a background measured from an area adjacent to the relevant band). Band intensities were expressed relative to control bands on the same gel.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Measurements of Angles of Mitotic Spindles and NuMA Crescents**

We used a published method to measure spindle angle relative to the substratum (Toyoshima et al., 2007; Toyoshima and Nishida, 2007). Briefly, confocal z stack images (0.5-μm thick) of samples stained for α- or γ-tubulin, or transfected with mCherry-α-tubulin, were projected as a 3D image to obtain a side view using FIJI software 3D project plugin. The distance between the mitotic poles (A) and the height difference between them (Z) was measured (spindle angle = ArcSin(Z/A)) (Figure 1A).

For NuMA crescent angles, the difference between the centers of each crescent in a z stack (Z<sub>1</sub>-Z<sub>2</sub>) and their separation in the x-plane (X) were measured (NuMA angle = ArcTan((Z<sub>1</sub>-Z<sub>2</sub>)/X)) (Figure S4E).

**Measurements of IP<sub>3</sub>R Distributions Around Mother and Daughter Centrosomes**

Mother and daughter centrosomes were distinguished by immunostaining for cenexin, which more intensely labels the mother centrosome (Colicino et al., 2019). The confocal section in which each centrosome was most intensely labeled was then used to analyze IP<sub>3</sub>R distribution (immunostained or EGFP) around that centrosome. For each section, the average fluorescence intensity (mean ± SD) was determined, and pixels surrounding each centrosome that exceeded a threshold (mean + 2SD) were used to calculate areas occupied by IP<sub>3</sub>Rs (Figure 3B).

**Measurements of Lengths of Astral Microtubules**

We used two different methods to measure the length of astral microtubules: tracing anti-α-tubulin staining from each centrosome (Figures 3C and 3D) and measuring distances from centrosomes to immunostained EB3 (Figures S4C–S4G). Cells immunostained for cenexin, α-tubulin and/or EB3 and/or with the plasma membrane identified by WGA-staining were imaged using spinning-disk confocal microscopy. After background correction, Z stack projections of five sections that included each centrosome were used to measure the lengths of astral microtubules in the area generated by projecting a line passing through each centrosome, perpendicular to the spindle axis using FIJI (Figure S4E). The same methods were used to quantify cortical EB3 by counting immunostained EB3 puncta associated with WGA.
Statistical Analyses
Statistical analyses used GraphPad Prism version 6. Results are presented as means ± SD or SEM, as appropriate. Paired or unpaired Student’s t tests (for 2 variables) or one-way ANOVA with Bonferroni post hoc test (for multiple comparisons) was used for statistical analyses (*p < 0.05, **p < 0.01 and ***p < 0.001). χ² test for trend was used for comparisons of frequency distributions. Sample sizes and the tests used are provided in figure legends.
Supplemental Information

Ca\textsuperscript{2+} Release by IP\textsubscript{3} Receptors Is Required to Orient the Mitotic Spindle

Raul Lagos-Cabré, Adelina Ivanova, and Colin W. Taylor
Figure S1. Cells Without IP_3Rs Proliferate Normally But With Aberrant Spindles. Related to Figure 1.

(A) Numbers of cells/well for cells plated on day 1 with 100,000 cells/well. Mean ± SD from 3 experiments.

(B) In normal cells, the metaphase plate aligns perpendicular to the substratum, with the mitotic spindle parallel to it.

(C) Spindle angles measured in fixed WT and HEK-IP_3R-KO cells using γ-tubulin staining to identify centrosomes. Individual values mean ± SD from 3 experiments. ***P < 0.001, Student’s t-test.

(D) Brightfield images show that both daughter cells remain attached to the substratum during anaphase of a WT cell, while in the HEK-IP_3R-KO cell only one daughter cell remains attached. Scale bars = 20 µm

(E) Image of a multinucleate HEK-IP_3R-KO cell stained with α-tubulin (red) and DAPI (blue). Scale bar = 20 µm.

(F) Summary shows numbers of multinucleate cells. Mean ± SEM, n = 8 experiments, with 10 cells analysed in each. *P < 0.05, Student’s t-test.

(G) Typical example of a multipolar spindle in a HEK-IP_3R-KO cell stained for α-tubulin (red), γ-tubulin (yellow) and DNA (blue). Scale bar = 10 µm.

(H) Summary shows numbers of cells with multipolar spindles. Mean ± SEM, n = 3 dishes with 11 fields quantified in each. **P < 0.01, Student’s t-test.
Figure S2. Spindle Angles in HAP1 Cells Without IP₃Rs and in HEK Cells Expressing Single IP₃R Subtypes. Related to Figure 1.
Legend on next page.
Figure S2. Spindle Angles in HAP1 Cells Without IP₃Rs and in HEK Cells Expressing Single IP₃R Subtypes. Related to Figure 1.

Figure on preceding page.

(A) Confocal z-stack images from fixed HAP1 cells show spindle angles during metaphase for a WT and HAP1-IP₃R-KO cell. The cells are immunostained for α-tubulin (red) and γ-tubulin (green), and stained with DAPI (blue) for chromosomes. Scale bars = 10 µm.

(B) Spindle angles for WT and HAP1-IP₃R-KO cells. Individual values, mean ± SD from 5 experiments. *P < 0.05, Student’s t-test.

(C) Frequency distribution of the spindle angles (data from panel B). *P < 0.05, relative to WT, χ² test for trend.

(D) Typical western blots, using antibodies selective for IP₃R subtypes (IP₃R₁-₃) or an antibody that recognizes all three IP₃R subtypes (all IP₃R), show expression of IP₃R subtypes in WT HEK cells, HEK-IP₃R-KO cells and cells expressing single native IP₃R subtypes (Alzayady et al., 2016). The results replicate our previous analyses, which established that the HEK cell line used expresses 36% IP₃R₁, 18% IP₃R₂ and 45% IP₃R₃ (Mataragka and Taylor, 2018).

(E) Spindle angles of cells expressing single IP₃R subtypes. Results show individual values, mean ± SD from 5 experiments. ***P < 0.001 relative to WT, ANOVA with Bonferroni test.

(F) Frequency distribution of spindle angles (data from panel B). ***P < 0.001, relative to WT, χ² test for trend.
Figure S3. IP$_3$R3 and EGFP-IP$_3$R3 Similarly Rescue Spindle Alignment. Related to Figure 1.

(A) Relationships between immunostaining for IP$_3$R3 and spindle angle measured under identical conditions for WT and HEK-IP$_3$R-KO cells, or the latter expressing IP$_3$R3 or EGFP-IP$_3$R3. Points are from individual cells taken from 5 experiments. AFU, arbitrary fluorescence units (note the ten-fold difference in scale for WT and IP$_3$R-KO cells relative to transfected cells). For IP$_3$R-KO cells, all cells contributed to the analysis; whereas for the other cells only those with detectable immunostaining were included. From least-squares linear regression, the only slope to differ significantly from 0 was for WT cells ($P < 0.0001$). The results suggest firstly that the level of IP$_3$R expression in WT cells may contribute to whether the spindle aligns appropriately, and secondly that even massive over-expression of IP$_3$R does not perturb spindle alignment. Summary results in Figures 1I and 1J.

(B) Cells heterologously expressing IP$_3$R3 were selected (by immunostaining) to approximately match WT in their expression of IP$_3$R3. Note that in WT cells IP$_3$R3 comprises only ~45% of the IP$_3$Rs expressed (Mataragka and Taylor, 2018), whereas IP$_3$R3 is the only subtype in heterologously-expressing cells. Results show relative expression levels of IP$_3$R3 for cells used in the analyses shown in panels C and D.

(C) Spindle angles measured in the indicated cells, approximately matched for their expression of IP$_3$R3 (see panel B). Individual values, mean ± SD. ****$P < 0.0001$, *$P < 0.05$, one-way ANOVA with Holm-Sidak multiple comparisons test, relative to HEK-IP$_3$R-KO cells.

(D) Distributions of spindle angles (data from panel C). ***$P < 0.001$, relative to WT, +$P < 0.05$ relative to IP$_3$R-KO, $\chi^2$ test for trend. Results shown here comprise a subset of the data (cells with IP$_3$R3 expression approximately matching expression in WT cells) shown in Figures 1I and 1J.
Figure S4. Analyses of Contact Areas, NuMA Angles and IP₃-Evoked Ca²⁺ Release. Related to Figures 2 and 4. Legend on next page.
Figure S4. Analyses of Contact Areas, NuMA Angles and IP₃-Evoked Ca²⁺ Release. Related to Figures 2 and 4.
Figure on preceding page.

(A) Confocal and TIRF microscopy was used to measure contact areas with the substratum for dividing cells with membranes labelled using DHCC (green). Scale bars = 10 𝜇m. Summary results in Figure 2A.

(B) Typical confocal sections and brightfield images of isolated synchronized WT HEK cell expressing mCherry-α-tubulin and with DNA stained with NucBlue. Scale bar = 10 𝜇m. Similar images were used to provide z-stacks from which spindle angles were measured (Figures 2B-D).

(C) NuMA links dynein to microtubules, and, through associated proteins, it associates with cortical actin. PCM, pericentriolar material.

(D) Examples of WT and HEK-IP₃R-KO cells in metaphase immunostained for NuMA. The large images show a single confocal section that includes a centrosome. Small images are sections (numbered from base of cell) showing locations of centrosomes (*) and the bounds of each NuMA crescent (red bars) and its centre (yellow bars, from which NuMA angles were calculated). NuMA angles were measured between the central points of the NuMA crescents relative to the substratum (Figures 2E and 2F). Scale bars = 10 𝜇m.

(E) NuMA angles relative to the substratum were calculated as shown. The z-planes in which the centre of each NuMA crescent was located were identified (Z₁ and Z₂) and the angle between them (α) was calculated.

(F) Location of the mutated residue (D2477 of IP₃R3) within the selectivity filter that is conserved within all three IP₃R subtypes and falls within the re-entrant luminal loop linking transmembrane domains (TMD) 5 and 6. IBC, IP₃-binding core.

(G) Typical western blot (20 μg protein/lane) using antibodies to IP₃R3 (top) or GFP (bottom) of WT HEK cells or HEK-IP₃R-KO cells expressing the indicated IP₃R3 proteins. In 4 independent paired analyses, the intensity of the IP₃R3 band (determined using an Ab for IP₃R3) for cells expressing EGFP-IP₃R3D/A was 81.2 ± 4.5% that from cells expressing EGFP-IP₃R3.

(H) Ca²⁺ signals recorded using Calbryte-590 from HEK cells transiently expressing EGFP-IP₃R3s. Carbachol (CCh, 100 μM) was added to stimulate IP₃ formation through endogenous muscarinic acetylcholine receptors. Ionomycin (100 μM) was added to allow saturation of the Ca²⁺-indicator. Results (F/F₀, where F is fluorescence recorded at each time, and F₀ fluorescence recorded at t = 0) are means from at least 22 successfully transfected cells from 3 experiments. The results establish that IP₃R3D/A does not mediate IP₃-evoked Ca²⁺ release (Boehning et al., 2001; Dellis et al., 2008).
Figure S5. Astral Microtubules in Cells With and Without IP3Rs. Related to Figure 3.
Legend on next page.
Figure S5. Astral Microtubules in Cells With and Without IP₃Rs. Related to Figure 3.

Figure on preceding page.

(A) Z-stacks from confocal sections that included both centrosomes (identified by cenexin staining) (Figure 3C) were used to measure lengths of astral microtubules at each pole of each cell (~8 measurements per cell). Results show mean values for each cell (without distinguishing mother and daughter centrosomes), and mean ± SEM for all cells. No significant difference between WT and HEK-IP₃R-KO cells, Student’s t-test.

(B) Typical confocal images (central plane) show ER (GFP-ER, green) and immunostained IP₃R3 (red) in interphase WT HEK cells and HEK-IP₃R-KO cells expressing IP₃R3. Scale bars = 10 µm. Results confirm the similar subcellular distributions of native and heterologously expressed IP₃R3.

(C) Confocal image of HEK-IP₃R-KO cell immunostained for EB3 (green) and α-tubulin (red), showing EB3 capping astral microtubules. Scale bars = 10 µm (5 µm in enlargement of boxed area).

(D) Maximum intensity z-stack images around centrosome of HEK cells immunostained for EB3 (green), α-tubulin (magenta) and cenexin (grey), and with the plasma membrane (PM) identified using wheat germ agglutinin (WGA, red). Arrows show EB3 at the cell cortex (PM). Scale bars = 10 µm (5 µm in enlargements of areas surrounding centrosomes). D, daughter; M, mother.

(E) Summary results show the fraction of EB3 puncta located at the PM around mother and daughter centrosomes (areas shown in the inset) for cells with (WT, n = 22 cells) or without IP₃Rs (IP₃R-KO, n = 20 cells). Results show individual values and mean ± SD. No significant difference between any values, one-way ANOVA.

(F) Maximum intensity z-stack images around centrosome immunostained for EB3 (red) and cenexin (grey) in HEK-IP₃R-KO cells transfected with EGFP-IP₃R3 or EGFP-IP₃R3DA. GFP-Booster was used to enhance the fluorescence of EGFP. Scale bars = 10 µm (5 µm in enlargements of regions surrounding centrosomes).

(G) Summary results show distance between centrosomes (cenexin) and EB3 in WT cells and HEK IP₃R-KO cells alone or after expression of EGFP-IP₃R3 or EGFP-IP₃R3DA. Results show individual values, mean ± SD. *P < 0.05, Paired student’s t-test, mother relative to daughter centrosome.

(H) Numbers of immunostained EB3 puncta (#/µm²) identified in regions around each centrosome (see inset to panel E). Results show individual values with mean ± SD from 22 (WT) or 20 (IP₃R-KO) cells. No significant differences, one-way ANOVA.