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REVIEW

# Microtubule-dependent subcellular organisation of pluripotent cells

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## ABSTRACT

With the advancement of cutting-edge live imaging technologies, microtubule remodelling has evolved as an integral regulator for the establishment of distinct differentiated cells. However, despite their fundamental role in cell structure and function, microtubules have received less attention when unravelling the regulatory circuitry of pluripotency. Here, we summarise the role of microtubule organisation and microtubule-dependent events required for the formation of pluripotent cells *in vivo* by deciphering the process of early embryogenesis: from fertilisation to blastocyst. Furthermore, we highlight current advances in elucidating the significance of specific microtubule arrays in *in vitro* culture systems of pluripotent stem cells and how the microtubule cytoskeleton serves as a highway for the precise intracellular movement of organelles. This Review provides an informed understanding of the intrinsic role of subcellular architecture of pluripotent cells and accentuates their regenerative potential in combination with innovative light-inducible microtubule techniques.

**KEY WORDS: Pre-implantation embryo, Microtubule cytoskeleton, CAMSAP3, Pluripotency, Stem cells, Organelles, Live imaging**

## Introduction

Pluripotency defines the remarkable ability of cells to become any cell type of the body. This unique feature is associated with the activation of key pluripotency proteins, including OCT4, SOX2 and NANOG, along with the functional properties of rapid proliferation and asymmetric division.

*In vivo*, the pre-implantation mammalian embryo presents a unique physiological model with which to unravel the morphological aspects of pluripotency (see Glossary, Box 1). Although pre-implantation development starts with the totipotent zygote, it is not until the 16-cell stage that inner and outer cells segregate to eventually form a blastocyst (see Glossary, Box 1) with an inner cell mass (ICM; see Glossary, Box 1) containing the pluripotent epiblast (see Glossary, Box 1). As embryo development proceeds from pre- to post-implantation stages, pluripotent cells transition from naïve to formative, before becoming primed immediately before gastrulation (Nichols and Smith, 2011; Shahbazi et al., 2017; Smith, 2017; Tam and Behringer, 1997). Throughout this progression, pluripotent cells undergo rapid proliferation and differential gene expression, and undertake major morphological changes to become increasingly specialised. Thus, pluripotency is not an abstract feature; instead, the formation of the pluripotent cells of the self-organised pre-implantation embryo could be dependent on the precise regulation of the subcellular architecture in alignment with metabolic and genetic factors. Pluripotency can also be studied *in vitro* by the isolation of cells of the ICM, termed embryonic stem cells (ESCs), or by cellular reprogramming (see Glossary, Box 1).

Deciphering the mechanisms that contribute to pluripotent cell identity has been a central goal across both discovery and clinical research. Although pluripotency has been extensively defined at genetic, epigenetic and metabolic levels, how subcellular dynamics contribute to the potency of cells has remained elusive until recently.

In this Review, we summarise the roles of microtubule organisation and microtubule-dependent subcellular processes in the pre-implantation embryo and in *in vitro* pluripotent stem cell (PSC) systems during interphase. Although we predominantly focus on mammalian studies, we make references to selected examples from other species. By highlighting research that has uncovered roles for organelle dynamics in pluripotency, we speculate on how the cytoskeleton-organelle interactome might be part of the pluripotency puzzle. Finally, we discuss the prospects of live imaging and cutting-edge optogenetics in elucidating the role of microtubules as integral regulators of pluripotency *in vivo* and *in vitro* (Guo et al., 2018; Julian and Stanford, 2020; Liu et al., 2018; Zenker et al., 2017).

## The cytoskeleton

The foundation for the spatiotemporal organisation of cells is provided by the cytoskeleton, a network of microtubule (see Glossary, Box 1), actin and intermediate filaments (Hohmann and Dehghani, 2019). To date, actin has formed the epicentre of cytoskeletal research in embryogenesis and tissue formation (reviewed by Shamipour et al., 2021). In contrast, the crucial role for microtubules in subcellular organisation beyond cell division (Akhmanova and Hoogenraad, 2015; Sanchez and Feldman, 2017) has been relatively understudied. Microtubules comprise α- and β-tubulin heterodimers defining the fast-growing plus and slow-growing minus ends (Gudimchuk and McIntosh, 2021). The former is characterised by a common mechanism of microtubule polymerisation and depolymerisation in various cell types (Mitchison and Kirschner, 1984). The latter, however, undertakes cell type-specific anchoring and protein interactions (Akhmanova and Hoogenraad, 2015).

Since the first visualisation of microtubules in living cells in the 1980s (Keith et al., 1981; Taylor and Wang, 1980), our understanding of the microtubule cytoskeleton has transformed drastically. The development of epi-fluorescence microscopy and fluorescent-labelling techniques makes it possible to continuously observe the behaviour of cytoskeletal proteins *in vivo* (Mitchison and Kirschner, 1984; Salmon et al., 1984; Taylor and Wang, 1980). These approaches have unveiled that, contrary to the word 'skeleton', microtubule filaments are not fixed but highly dynamic, with the ability to rapidly adapt to the needs of a cell. More recently,

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## Box 1. Glossary

**Abscission.** The severing of the cytokinetic bridge connecting two daughter cells at the end of cytokinesis. The abscission site is determined by the midbody, a transient structure comprising dense microtubules, which are remnants of the mitotic spindle.

**AKAP450.** A-kinase anchoring protein 450, also known as AKAP9 and CG-NAP, is a scaffolding protein interacting with the centrosomal γ-tubulin ring complex. Recruitment of AKAP450 to the Golgi apparatus is essential for microtubule nucleation. In addition, binding of AKAP450 to CAMSAP3 mediates anchoring and elongation of microtubules, and the positioning of the Golgi in epithelial cells.

**Blastocyst.** The mammalian blastula; a pre-implantation developmental stage when the embryo is composed of a fluid-filled cavity (blastocoel) and two cell layers: the trophectoderm and the inner cell mass (ICM).

**CAMSAP3.** Calmodulin-regulated spectrin-associated protein family member 3, also known as Nezha or Marshalin. The CAMSAP protein family includes three members (CAMSAP1, CAMSAP2 and CAMSAP3) in vertebrates and a single member in invertebrates: patronin. Binding of CAMSAPs and/or patronin to microtubule minus ends protects filaments from depolymerisation.

**Cellular reprogramming.** Reversion of specialised somatic cells into induced pluripotent stem cells.

**CKK domain.** A C-terminal protein domain, specific to the CAMSAP and patronin proteins that binds to the minus end of microtubules. The domain was originally identified in CAMSAP1, KIAA1078 (CAMSAP2) and KIAA1543 (CAMSAP3), is conserved in all eumetazoan and has a β-barrel with an associated α-helical hairpin structure.

**Epiblast.** In mammalian embryos, the epiblast is one of two cell layers derived from the inner cell mass. The epiblast forms the embryo proper through differentiation into the three germ layers – meso-, endo- and ectoderm – during gastrulation.

**Inner cell mass.** One of the two cell layers of the blastocyst that goes on to develop two distinct cell layers: the pluripotent epiblast and the primitive endoderm or hypoblast.

**Intercellular microtubule bridges.** A bundle of microtubule filaments connecting cells during interphase. Such bridges were observed in both the mouse pre-implantation embryo and naïve ESCs. However, only in the embryo are they known to function as a ncMTOC: the interphase bridge.

**Microtubules.** Components of the tripartite cytoskeleton, comprising heterodimers of α- and β-tubulin subunits arranged in a 'head-to-tail' manner to form polarised polymers.

**Microtubule organising centre (MTOC).** A subcellular site for microtubule nucleation. There are two types of MTOC: (1) the centrosome, an organelle comprising two centrioles (a paired cylindrical-shaped organelle characterised by the arrangement of nine triplet microtubules) surrounded by the pericentriolar matrix (an electron-dense protein matrix); and (2) non-centrosomal MTOCs, which are found at various subcellular sites and facilitate diverse asymmetric microtubule networks.

**Motor proteins.** Cytoskeleton-associated protein complexes organising the position of intracellular structures by ATP hydrolysis. Two superfamilies are associated with microtubules: kinesins and dyneins. The latter move towards the microtubule minus ends.

**Pluripotency.** The ability of a cell to develop the three germ layers – ecto-, meso- and endoderm – and the germ line, but not to self-organise into a fertile adult individual.

**Post-translational modification.** Microtubule function and dynamics can be influenced by post-translational modifications. Certain post-translational modifications are reported in both α-/β-tubulin subunits, i.e. (de)acetylation, (de)glutamylation, (de)polyglutamylation, ubiquitylation, phosphorylation, glycation and glycososylation, whereas others are restricted to α-tubulin: methylation, palmitoylation, sumoylation and (de)tyronisation.

**Trophectoderm.** The epithelial-like outer layer of the blastocyst that mediates embryo implantation into the maternal tissue and is a precursor of the placenta lineage.

**γ-Tubulin ring complex.** A multiprotein complex essential for the function of the centrosome, which has γ-tubulin as a core component.

advances in super-resolution microscopy have enabled single microtubule filaments to be identified within complex cytoskeletal networks, allowing the precise characterisation of microtubule dynamics in more detail than ever before (Guo et al., 2018). Likewise, innovative live imaging is rapidly uncovering pivotal roles for microtubule-dependent processes, significantly reshaping our knowledge of subcellular architecture in real-time (Guo et al., 2018; Valm et al., 2017). In particular, the formation of highly diverse non-centrosomal microtubule networks has emerged as a fundamental requirement for the morphological and functional specialisation of differentiated cells (Akhmanova and Hoogenraad, 2015). Recently, increasing evidence supports the concept of a distinctive microtubule network in pluripotent cells. For example, the identification of intercellular microtubule bridges (see Glossary, Box 1) as facilitators of asymmetric intra- and intercellular transport demonstrates a pivotal role for microtubules in the regulation of pluripotency (Chaigne et al., 2020; Zenker et al., 2017). Despite this considerable progress, how the various cellular components, the cytoskeleton and organelles are arranged during the earliest stages of life remains a challenge to define.

## Microtubule organising centres and cell potency

The spatiotemporal configuration of microtubules is controlled by specialised subcellular microtubule organising centres (MTOCs; see Glossary, Box 1), which anchor the microtubule minus ends to enable microtubule outgrowth (Sanchez and Feldman, 2017). Conventionally, the centrosome (see MTOC in Glossary, Box 1) forms the primary MTOC in most multipotent, dividing eukaryotic cells. In this setting, the γ-tubulin ring complex (γ-TuRC; see Glossary, Box 1), surrounded by the pericentriolar matrix (PCM; see MTOC in Glossary, Box 1) serves as template for microtubule nucleation in a radial and symmetric fashion towards the cell periphery (Conduit et al., 2015).

During the transition into mature differentiated cells, the centrosome is (at least partially) inactivated. Instead, various other subcellular structures assume roles as non-centrosomal MTOCs (ncMTOCs; see MTOC in Glossary, Box 1), depending on the cell type (Table 1) (Martin and Akhmanova, 2018; Sanders and Kaverina, 2015), to regulate organelle positioning, communication and cell polarity (Tillery et al., 2018; Toya et al., 2016). This structural variability ultimately leads to diverse cellular functions (Table 1).

Calmodulin-regulated spectrin-associated protein 3 (CAMSAP3; see Glossary, Box 1) is a protein that is able to nucleate microtubules independently of γ-tubulin at ncMTOCs, facilitated by its evolutionarily conserved CKK domain (see Glossary, Box 1), which contains two additive microtubule interaction sites (Fig. 1). CAMSAP3 serves to shape parallel microtubule arrays growing along the apical-basal axis of epithelial cells during interphase (Meng et al., 2008). Similar to epithelial ncMTOCs, CAMSAP3 can also bind to AKAP450 (see Glossary, Box 1 and Table 1), potentially enabling CAMSAP3 to nucleate microtubules (Wang et al., 2017). Stabilisation of CAMSAP3-decorated microtubules might be required for counteracting kinesin 13 depolymerisation events and interactions with the microtubule-severing protein katanin (Dong et al., 2017; Jiang et al., 2018). However, the function of CAMSAP3 is considerably more versatile than the interplay with microtubules and microtubule-associated proteins, judging by its interaction partners and multi-domain construction (Akhmanova and Hoogenraad, 2015). Indeed, the name of CAMSAP3 reflects its competitive binding with spectrin and calcium/calmodulin, the biological relevance of which remains to be further investigated. Another calcium-dependent protein is the

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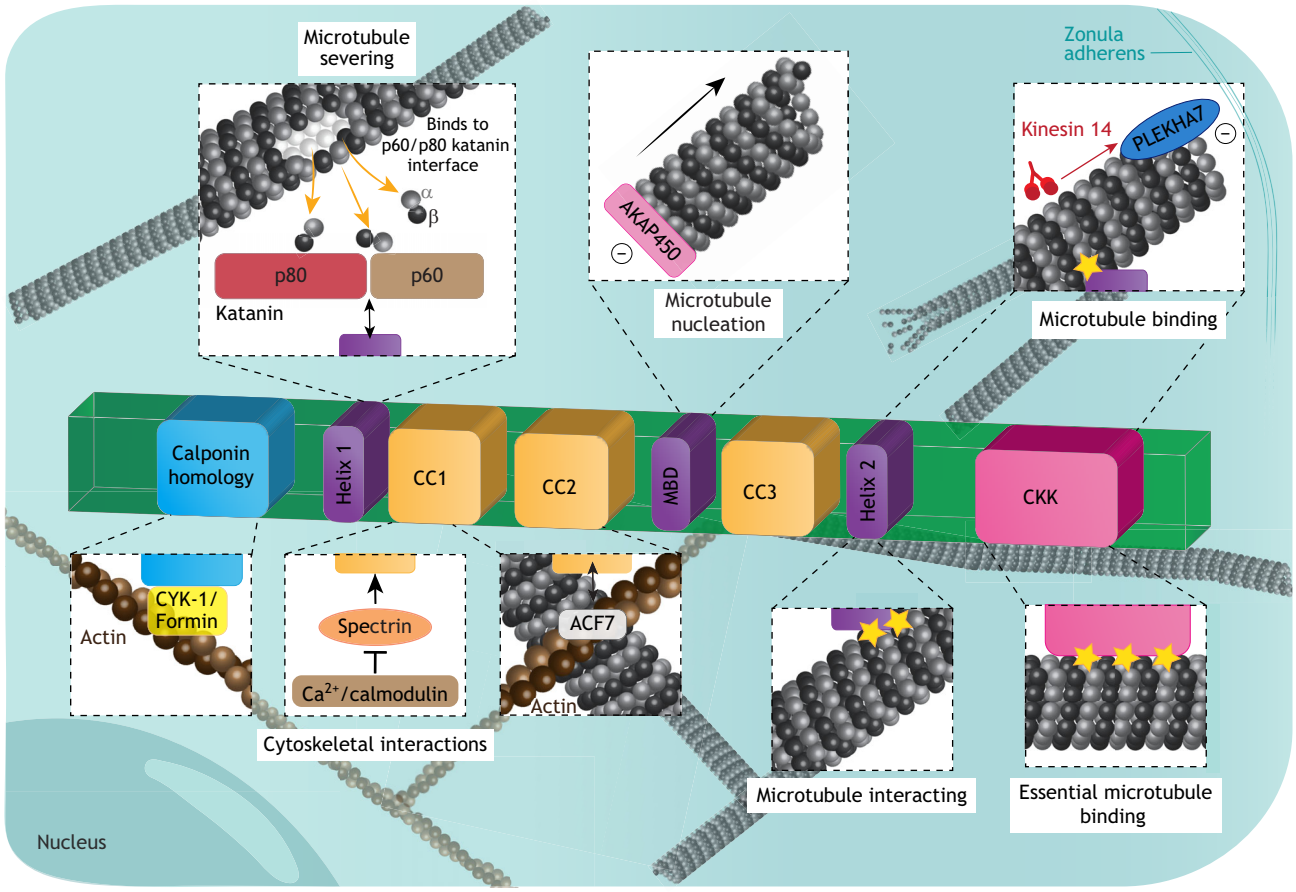


**Fig. 1. The multi-domain structure of CAMSAP3.** CAMSAP3 comprises eight domains facilitating a multitude of interactions. The N-terminal calponin homology domain binds to the actin nucleator formin (also known as CYK-1), followed by helix 1, which promotes katanin-dependent microtubule severing of α- and β-tubulin subunits from the microtubule protofilament. Eponymously, calcium and/or calmodulin competes with spectrin, a large cytoskeleton scaffold protein below the plasma membrane, to bind to the coiled coil 1 (CC1) domain of CAMSAP3. The microtubule binding capacity of CAMSAP3 starts at the CC1/CC2 domain by interacting with ACF7, which crosslinks microtubule (grey) and actin (brown) filaments. Binding of AKAP450 at the microtubule-binding domain (MBD) might permit microtubule nucleation, whereas helix 2 enhances microtubule binding (yellow stars). The C-terminal CKK domain masters the binding of CAMSAP3 with microtubules (yellow stars). PLEKHA7 also binds to the C-terminus of CAMSAP3, regulating its apical localisation and kinesin 14-dependent transport.

cell-adhesion molecule cadherin, which also interacts with CAMSAP3, potentially indicating a crucial requirement for calcium at CAMSAP3-dependent ncMTOCs (Takahashi et al., 2016). Similar to the cadherin-adhesion complex, CAMSAP3 is crosslinked to the actin cytoskeleton via a spectraplakin family member, ACF7 (Ning et al., 2016), and at the zonula adherens via PLEKHA7 (Meng et al., 2008). CAMSAP3 also binds to CYK-1 (also known as formin), a nucleator of filamentous actin, thus CAMSAP3 influences actin dynamics, in addition to microtubules (Fig. 1) (Wong et al., 2018).

In 1984, it was demonstrated for the first time that microtubules can be imaged in living sea urchin embryos (Salmon et al., 1984). Subsequently, this field has been dominated by research visualising microtubules during early cleavage stages and understanding the function of the centrosome. However, in many species, including ctenophores, nematodes, echinoderms, molluscs, ascidians, fish, amphibians and most mammals, the centrosome is degenerated in the oocyte (Clift and Schuh, 2015; Schatten, 1994; Schatten et al., 1991) and thus asymmetrically passed on from the spermatozoa upon fertilisation. This mechanism serves to circumvent the presence of two centrosomes and ensures subsequent progression of development (Chatzimeletiou et al., 2005). Although similarities in this process exist across species, they are not universal.

In *Mus musculus* (mouse), the early stages of embryogenesis occur in the absence of centrosomes, which instead form *de novo* later during development (Schatten, 1994) (Fig. 2A). Initially, microtubule organisation in the early mouse embryo was thought to be randomly organised (Howe and FitzHarris, 2013), a compelling reasoning that was further supported by the non-centrosomal localisation of canonical centrosome markers, such as PCM1 (Houliston et al., 1987). In addition, embryos lacking tubulin γ1 (TUBG1), a potent microtubule nucleator of the γ-TURC, persist until blastocyst stage (Yuba-Kubo et al., 2005). This argument, however, is not infallible as immunofluorescence and immunoelectron microscopy have revealed the presence of aligned microtubule arrays across sister blastomeres during interphase (Houliston et al., 1987; Kidder et al., 1988). As such, recent cutting-edge live imaging has uncovered functional ncMTOCs in the early mouse embryo (Zenker et al., 2017). Following mitosis, the typical cytokinetic bridge does not undergo abscission (see Glossary, Box 1), but instead is converted into an interphase bridge that is retained from the two-cell stage until at least the blastocyst stage (Fig. 2A; Fig. 3A). This structure is now defined as a ncMTOC in the mammalian embryo and recruits CAMSAP3. In the mouse embryo, CAMSAP3 directs the transport of E-cadherin, a cell-adhesion molecule required for embryonic development (Kan et al., 2007; Riethmacher et al.,

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**Table 1. Active microtubule organising centres in different cell types**

| Cell types | Subcellular site | Factors involved | References |
|---|---|---|---|
| Progenitor cells, migratory cells and cultured fibroblast | Centrosomal | γ-TuRC; centriolar proteins, including centrin 2 and SAS-6; PCM, including pericentrin, AKAP450 and ninein | Conduit et al. (2015) |
| Myotubule and striated muscle cells | Nuclear envelope | PCM1, AKAP450, γ-tubulin, PCNT, ninein and Cep215 | Becker et al. (2020); Musa et al. (2003) |
| Epithelial cells | Apical membrane | CAMSAP3, ACF7 and AKAP450 | Meng et al. (2008); Toya et al. (2016); Wang et al. (2017) |
| Neuron | Golgi apparatus | AKAP450, CAMSAP2, γ-tubulin and CLASP1/2 | Martin and Akhmanova (2018); Sanders and Kaverina (2015); Ye et al. (2007) |
| Spindle noncentrosomal | Cytoplasmic/ spindle | Augmin and γ-TuRC | Goshima et al. (2008) |
| *Drosophila* germ cells | Plasma membrane | γ-Tubulin | Lerit and Gavis (2011) |
| *Drosophila* spermatid | Mitochondria derivatives | Cnn and γ-TuRC | Chen and Chan (2017); Tillery et al. (2018) |

1995), to the membrane, and downregulation of CAMSAP3 reduces the capacity of the embryo to form pluripotent cells (Zenker et al., 2017). Whether this reduction is co-triggered by the ability of CAMSAP3 to regulate Yes-associated protein (YAP) activity (Mitsuhata et al., 2021), which is a crucial transcription factor required for lineage segregation in the mammalian embryo, remains to be determined. Given the large number of CAMSAP3 interactors (Fig. 1), its full potential in regulating early mammalian embryogenesis may not yet be recognised. In fact, interphase bridge-like structures are also visible in fixed pre-implantation human embryos labelled by α-tubulin (Chatzimeletiou et al., 2005). In humans, paternally inherited centrioles (see MTOC in Glossary, Box 1) are present in the embryo (Avidor-Reiss, 2018; Fishman et al., 2018), but whether they actively participate in microtubule nucleation or organisation during interphase remains a point of contention. Similarly, in *Xenopus laevis* and *Danio rerio* (zebrafish) embryos, bridges composed of tubulin at the cleavage furrow initiate the formation of radial microtubules growing outwards into the cell periphery (Hasley et al., 2017; Eno et al., 2018) (Fig. 2B; Fig. 3B). These furrow-associated microtubule arrays are independent of the midzone and spindle microtubules, and are orthologous to the mammalian midbody associated with abscission (Danilchik et al., 1998; Jesuthasan and Stähle, 1997; Otegui et al., 2005). Overall, these findings support a role for microtubule bridge-like structures as conserved ncMTOCs driving early embryogenesis.

A centrosome-independent subcellular microtubule organisation might also exist in *D. melanogaster*. In oocytes and embryos in which DSAS4, the *Drosophila* ortholog of spindle assembly defective protein 4 (SAS-4), is mutated, larvae die shortly after hatching (Basto et al., 2006). Early development is sustained instead by the heterozygous maternal contribution of DSAS-4 to unfertilised oocytes (Basto et al., 2006). Similarly, the absence of core centrosomal component centrosomin (Cnn), which is also known to initiate non-centrosomal microtubule formation on mitochondria (Table 1), results in developmental arrest in all progeny of *D. melanogaster* Cnn mutants (Vaizel-Ohayon and Schejter, 1999). These findings question whether the centrosome acts as the only MTOC during early *D. melanogaster* embryogenesis. Furthermore, live imaging of homogenised interphase-arrested *X. laevis* egg extracts shows rapid re-organisation into cell-like structures independent of sperm nuclei or the translation of pre-existing maternal mRNA (Cheng and Ferrell, 2019). Instead, microtubule polymerisation and cytoplasmic threshold concentration can restore cellular organisation and reproductive function (Cheng and Ferrell, 2019). Collectively, these findings are indicative of microtubule subcellular organisation beyond the centrosome. However, despite the important roles of non-centrosomal microtubules, the exact details of why they are needed, how they function and how they regulate the localisation of cellular components require further elucidation.

## Emerging roles of the microtubule cytoskeleton as a regulator of early embryogenesis

### Fertilisation

As the oocyte transitions into a zygote, large-scale cytoplasmic reorganisation occurs. Despite the lack of centrosomes, the mouse zygote contains acentriolar microtubule-organising centres (Fig. 2A) and a complex microtubule network (Schatten, 1994). In association with motor proteins (see Glossary, Box 1), this network coordinates cytoskeletal restructuring that regulates the transport of molecules, determines pronuclear migration and the cleavage plane of cells (Scheffler et al., 2021). After fertilisation, maternal and paternal pronuclear movement is coordinated via an actin- and microtubule-mediated, two-step mechanism that has been uncovered by live cell confocal and super-resolution microscopy (Scheffler et al., 2021). First, flattening of the fertilisation cone is followed by the local nucleation of actin by spire and formin 2 in a Rab11a-dependent manner, which pushes the male pronucleus away from the membrane (Scheffler et al., 2021). Second, the microtubule network recruits cytoplasmic dynein (see motor proteins in Glossary, Box 1) to centre the maternal and paternal pronuclei (Fig. 4A) (Scheffler et al., 2021). Similarly, in nematodes, echinoderms and amphibians, microtubule asters from paternally inherited centrosomes mediate pronuclear movement in concert with dynein, which is thought to establish cortical links between microtubules and the cortex, or by generating cytoplasmic forces (Fig. 2C) (Gönczy et al., 1999; Kimura and Kimura, 2011; Meaders and Burgess, 2020; Wühr et al., 2010). Thus, it has been proposed that cytoplasmic reorganisation might be independent of the centrosome and, instead, non-centrosomal microtubule organisation might meet the requirements for large-scale movements during this process (Ishihara et al., 2014; Kimura and Kimura, 2011; Meaders and Burgess, 2020; Nishikata et al., 2019; Wühr et al., 2010). Furthermore, after fertilisation of the *D. rerio* oocyte, the cytoskeleton regulates the segregation of the ooplasm into the yolk cell and the blastodisc domains (Hart and Fluck, 1996). Short dynamic microtubules located in the yolk cell are reorganised during the first cell cycle into a dense parallel array at the vegetal

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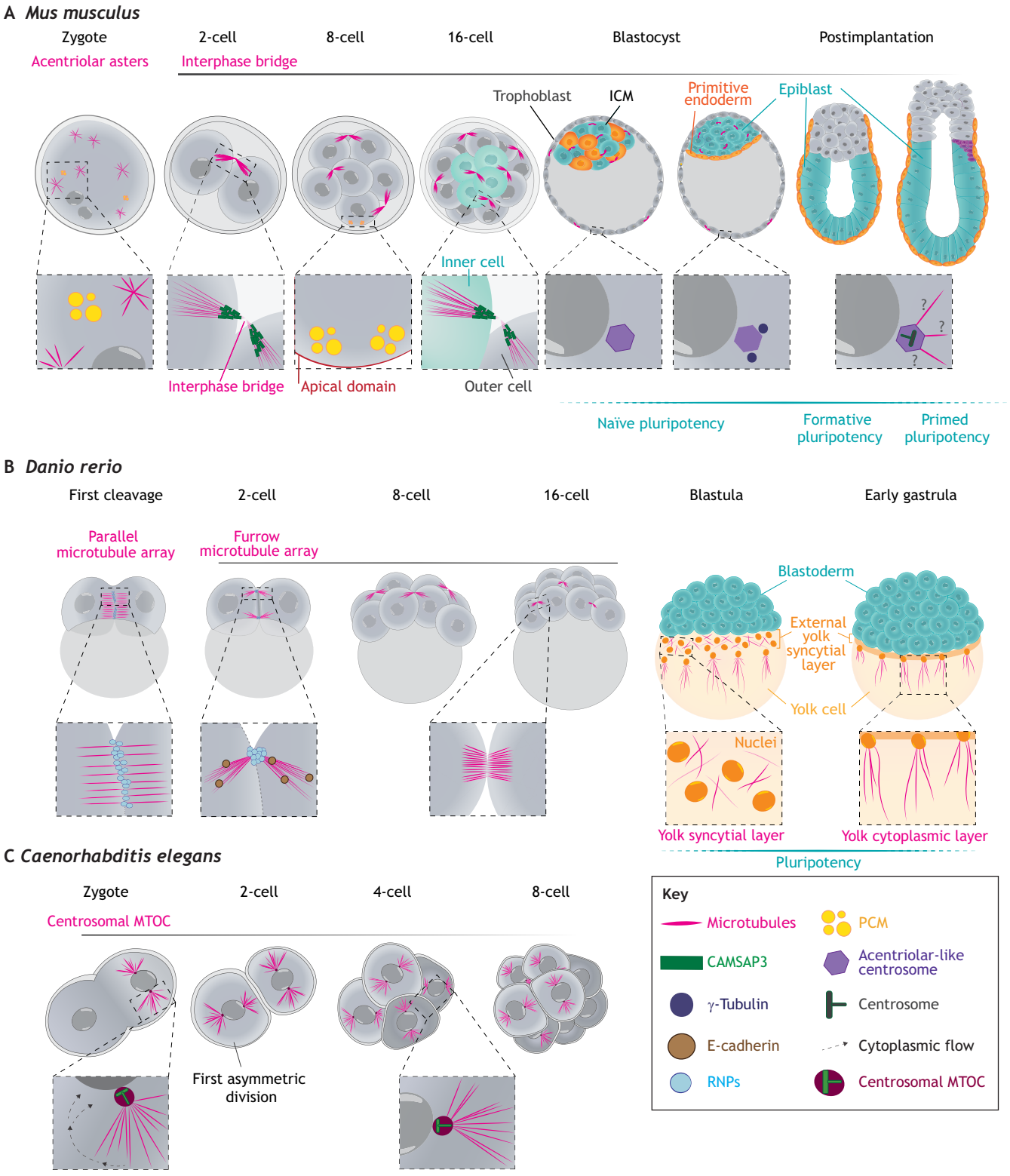


**Fig. 2. Species-specific overview of microtubule organisation during early embryogenesis.** (A) Microtubule organisation in the mouse embryo from the zygote to early post-implantation stages. The interphase bridge serves as a non-centrosomal microtubule organising centre (ncMTOC) throughout pre-implantation development. Centrosomes first emerge at the 16- to 32-cell stage, but they do not emanate microtubules or influence the interphase microtubule network. Centriole-containing centrosomes might be essential around gastrulation, suggesting the presence of a dual microtubule network. (B) In the *D. rerio* zygote, parallel microtubules accumulate ribonucleoparticles (RNPs) at the cleavage furrow. From the two-cell stage until around the 64-cell stage, furrow-associated microtubule arrays connect sister cells. As the embryo reaches the blastula stage, two interphase microtubule arrays are present and persist until the early gastrula stage. The yolk syncytial layer consists of intercrossing microtubules, and, at the yolk cytoplasmic layer, microtubules extend proximal to the nucleus in the direction of epibolic movement. (C) Early embryogenesis in *C. elegans* occurs in a centrosomal manner, whereby microtubules nucleate from the centrosomes. CAMSAP3, calmodulin-regulated spectrin-associated protein 3; ICM, inner cell mass; MTOC, microtubule-organising centre; PCM, pericentriolar matrix.

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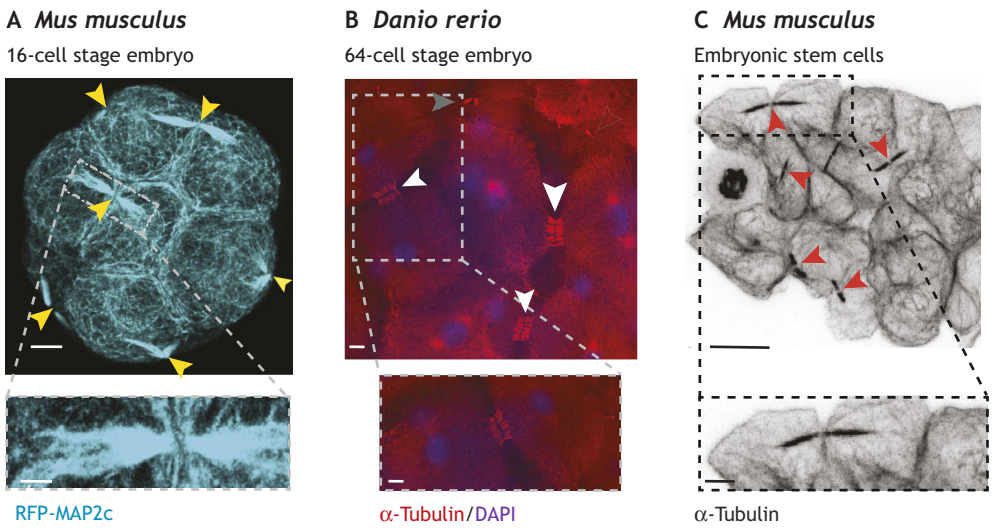


**Fig. 3. Species-specific non-centrosomal microtubule structures in the embryo and *in vitro* PSCs.** (A) Live imaging of the mouse pre-implantation embryo labelled for the fluorescently tagged microtubule-associated protein 2c (RFP-MAP2c; cyan) reveals intercellular microtubule bridges during interphase (yellow arrowheads). Reproduced, with permission, from Zenker et al. (2017). (B) Fixed 64-cell stage *D. rerio* embryo, stained for α-tubulin (red) and with DAPI (blue) reveals inward bundling furrow-associated microtubule arrays (white arrowheads) and remnants (grey arrowhead between sister cells). Adapted from Eno et al. (2018), where it was published under a CC-BY 4.0 license. (C) Fixed naïve mouse embryonic stem cell colony stained for α-tubulin (black) demonstrates prominent cytoplasmic bridges (red arrowheads) regulating naïve pluripotency exit. Adapted from Chaigne et al. (2020), where it was published under a CC-BY 4.0 license. Scale bars: 10 µm; 2 µm for insets in A-C.

pole (Jesuthasan and Stähle, 1997). This microtubule arrangement establishes the initial asymmetries in the embryo that specifies the dorsoventral axis by the transfer of substances such as ribonucleoparticles.

### Establishing polarity

Major morphological changes occur during polarisation and compaction of the mammalian embryo. Despite conveying intrinsic structural polarity, the roles of the microtubule cytoskeleton during these processes remain less defined. As polarisation occurs, distinct apical and basolateral domains are established in the blastomeres of the embryo. The apical surface is characterised by the localisation of the evolutionarily conserved Par3-Par6-aPKC complex, whereas E-cadherin, Jam-1, $Na^+/K^+$-ATPase and Par1 are present on the opposite basolateral side (Yamanaka et al., 2006).

At the early eight-cell stage in mice, scanning transmission electron microscopy has demonstrated that microtubules are distributed around the nucleus and cell cortex (Ducibella, 1982; Houliston et al., 1987). As development progresses, tyrosinated microtubules are highly enriched at the apical domain (Houliston and Maro, 1989) and acetylated tubulin at the basal domain (Fig. 4B) (Houliston and Maro, 1989). In other species and cell types, cytoskeletal rearrangements and redistribution of cytoplasmic structures are also involved in polarisation (Ducibella et al., 1977; Fleming et al., 1986; Houliston et al., 1987). For example, in neurons, microtubules are stabilised via acetylation that specifies polarisation (Witte et al., 2008). Furthermore, also in neurons, CAMSAP3-anchored microtubules prevent tubulin acetylation (Pongrakhananon et al., 2018). This therefore raises the question of whether CAMSAP3 may contribute to post-translational modifications (see Glossary, Box 1) in the early mouse embryo, because CAMSAP3 is located predominantly at the apical membrane in proximity to tyrosinated microtubules. The impact of CAMSAP3 on post-translational modifications, however, has yet to be unravelled (Tanaka et al., 2012). Similar processes occur in mammalian epithelial cells, where biased intracellular transport occurs towards non-centrosomal microtubules anchored at the apical membrane via CAMSAP3, which establishes and maintains polarity (Pongrakhananon et al., 2018; Toya et al., 2016). In *D. melanogaster* epithelial cells, polarity is established via a dynein-dependent transportation of mRNAs along microtubules to specific apical locations (Horne-Badovinac and Bilder, 2008). Furthermore, in *Caenorhabditis elegans* zygotes, the dense microtubule network located at the sperm centrosome limits PAR protein interactions, which results in an asymmetric organisation of polarised domains at the cell cortex (Labbé et al., 2003; Motegi et al., 2011). Several aspects of PAR protein self-organisation are proposed to partially involve microtubule-directed transport and targeting or inhibition of specific polarity regulators (Houliston et al., 1989; Motegi et al., 2011; Siegrist and Doe, 2007; Vinot et al., 2005). Collectively, these findings suggest a crucial role for microtubules in establishing cellular polarity during embryonic development that warrants further investigation.

Concomitant with polarisation is the process of compaction, where spherical blastomeres fortify their cell-cell contacts and adhere to each other. In addition to signalling cascades (Zhu and Zernicka-Goetz, 2020), the initiation and maintenance of compaction is tightly regulated by cytoskeletal forces (Maître, 2017; White et al., 2017), including microtubules running parallel to cell junctions that act to stabilise the membrane and to maintain the compacted state of the embryo (Ducibella, 1982). More recently, live imaging has uncovered E-cadherin-dependent cellular appendages, called filopodia, that extend on top of neighbouring cells to initiate compaction (Fierro-González et al., 2013). Manipulation by laser ablation has demonstrated that disturbance of these filopodia prevents compaction (Fierro-González et al., 2013). Although composed of actin filaments, microtubules are known to directly target filopodia and to alter their dynamics in fibroblasts and B16F1 melanoma cells (Schober et al., 2007). At the furrow-associated microtubule arrays in *D. rerio* and *X. laevis* embryos, microtubules mediate the directional transport of E-cadherin to the basement membrane (Danilchik et al., 1998;

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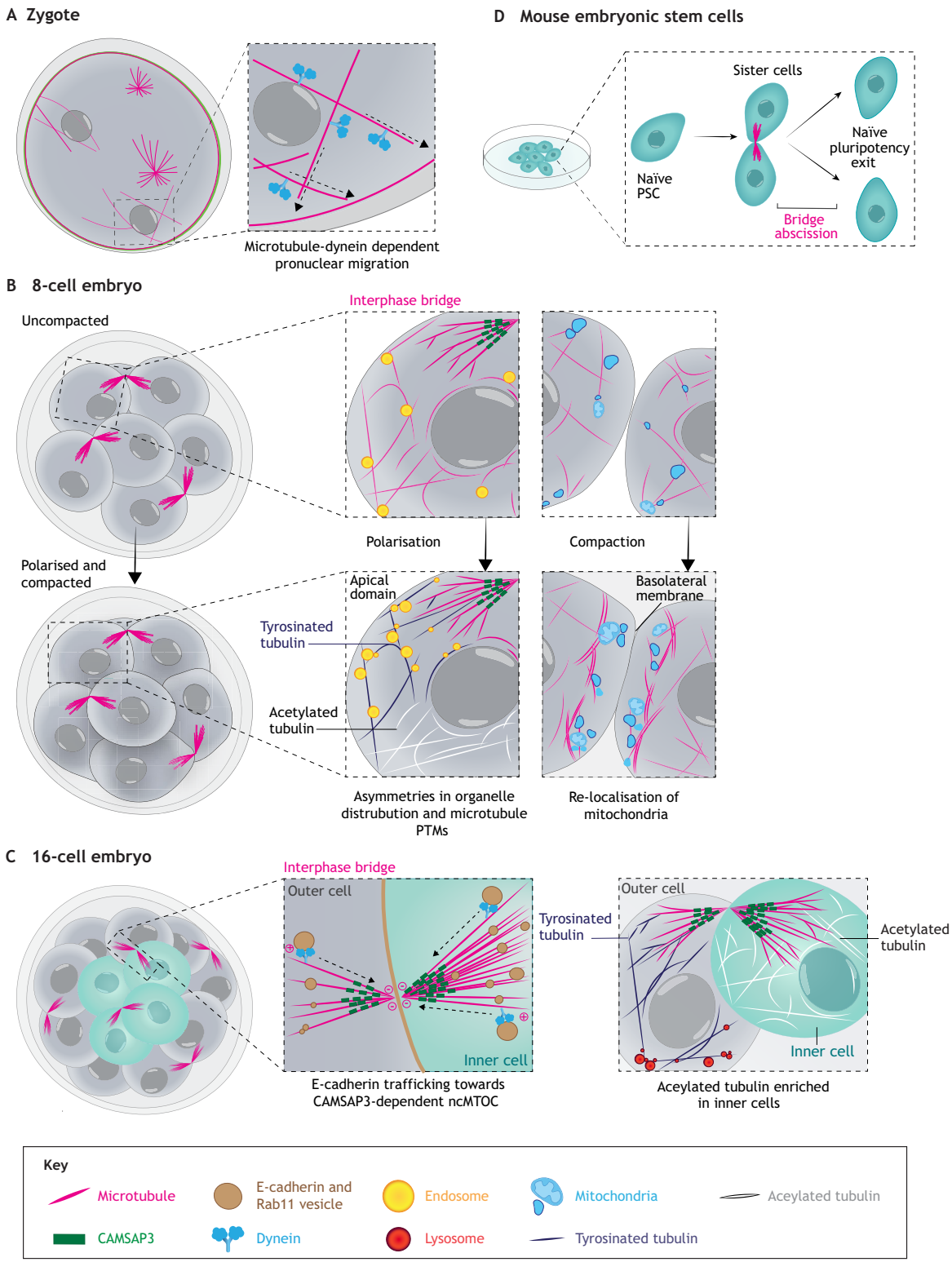


**Fig. 4. Microtubule-dependent mechanisms during mouse early embryogenesis and PSCs.** (A) Pronuclear centralisation in the mouse zygote is coordinated by microtubule-dynein-dependent movements. (B) Blastomeres of an eight-cell stage embryo undergo microtubule-dependent subcellular reorganisation during polarisation by localising tyrosinated tubulin and endosomes to the apical domain while acetylated tubulin dominates in the basolateral domain. During embryo compaction, mitochondria are concentrated and anchored to the basolateral membrane by a dense microtubule network. (C) At the 16-cell stage, the interphase bridge controls the asymmetric transport of E-cadherin towards the basolateral membrane. Accordingly, an increase in CAMSAP3-mediated non-centrosomal microtubule outgrowth on the side of the bridge of inner cells when compared with outer cells is essential for the formation of the ICM. In addition, acetylated tubulin is enriched in inner cells and tyrosinated tubulin is enriched in outer cells, where lysosomes adopt a basolateral location. (D) Mouse naïve embryonic stem cells undergo bridge abscission upon exit from naïve pluripotency. CAMSAP3, calmodulin-regulated spectrin-associated protein 3; ncMTOC, non-centrosomal microtubule organising centre; PSC, pluripotent stem cell; PTM, post-translational modifications.

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**Box 2. Cross-species comparison of microtubule regulation of cell differentiation**

The closest equivalent to pluripotent cells in *D. melanogaster* might be the imaginal discs (Maves and Schubiger, 2003). To evaluate whether the ability of disc cells to change their fate, called transdetermination, might be comparable with pluripotency is beyond the scope of this Review. In *X. laevis* embryos, pluripotent cells corresponding to the epiblast reside in the animal cap region during the mid-blastula stage. *D. rerio* embryos have a very short window of pluripotency from zygotic genome activation until oblong stage/late blastula (> ∼1000 cells) (Fig. 2B). Gene expression analysis has defined that differentiation of cell lineages in *D. rerio* occurs as early as the oblong stage (3.9 h post fertilisation). At ∼512 cells, the blastodisc contains pluripotent cells and the yolk cell might correspond to mammalian primitive endoderm cells (Paranjpe and Veenstra, 2015). Lineage tracing of cells using time-lapse photographic analysis has revealed that marginal cells of the blastoderm remain pluripotent throughout the late blastula to early gastrula stages (Ho and Kimmel, 1993).

Jesuthasan, 1998; Miller et al., 1993). The furrow-associated microtubule arrays recruit germplasm ribonucleoparticles to the furrow through their association with the astral ends of microtubules (Fig. 2B), which facilitates germ mass compaction and gives rise to primary undifferentiated stem cells (Eno et al., 2018; Jesuthasan, 1998). Notably, *D. rerio* embryos lacking the cytoskeletal protein gene *aur*, which encodes Mid1 interacting protein like 1 (Mid1ip1l), fail to recruit ribonucleoparticles to the furrow (Eno et al., 2018). Microtubule reorganisation and ribonucleoparticle recruitment has been suggested to supersede cell divisions for the determination of cell fate (Eno et al., 2018). Together, these findings indicate the importance of microtubule organisation for polarisation, compaction and the localised recruitment of subcellular components between daughter cells across species.

#### First lineage segregation

In mice, the 16-cell stage is marked by the generation of two morphologically and spatially distinct cell lineages of different developmental potential. The cells residing in the inside of the mammalian embryo form the ICM. In contrast, cells positioned at the outside develop into extra-embryonic trophectoderm (see Glossary, Box 1). Microtubule-dependent processes are pivotal for the morphological transformation, generating asymmetries between the inner and outer blastomeres by regulating the orientation of cleavage divisions, cell shape, polarity and nuclear movements (Ajduk et al., 2014; Fleming, 1987). Furthermore, microtubule organisation can bias transport towards subcellular locations. This can be directed by regions of higher microtubule density or by post-translational modifications to selectively recruit motor proteins for directed transport to one side of the cell (Burute and Kapitein, 2019). Microtubule density and acetylation are enriched in inner cells of the mouse embryo, whereas dynamic tyrosinated tubulin is more prominent in outer cells (Fig. 4C) (Houliston and Maro, 1989; Zenker et al., 2017). These asymmetries in microtubule modifications, established at the late eight-cell stage in mice, might be reinforced during the first cell fate decision in 16-cell stage embryos (Fig. 4B,C). Further to this, the microtubule interphase bridge projecting into an inner cell is more dense in microtubules and accumulates more CAMSAP3 than its outer sister cell (Zenker et al., 2017). Although the identity of post-translational modifications at the interphase bridge awaits elucidation, asymmetries in microtubule density in this setting bias intracellular transport. For example, an increased transport rate of E-cadherin and Rab11a vesicular-like structures towards the CAMSAP3-dependent ncMTOC facilitates E-cadherin integration into the membrane of unpolarised inner cells, lacking an apical domain (Fig. 4C). In outer cells of the 16-cell stage mouse embryo, a dense network of highly dynamic microtubules lies underneath apical actin rings (Zenker et al., 2018). After their *de novo* formation, cortical actin flows meet the growing microtubule plus ends, which expand the actin rings outwards to cell-cell junctions and seal the embryo to enable progression to blastocyst stage. However, how the intracellular organisation of inner and outer cells changes due to these cortically focused forces have not yet been revealed.

The segregation of the ICM is of fundamental significance for early embryogenesis; however, a comparison across species is exceedingly difficult (Box 2). Consequently, how microtubules might contribute to cell differentiation in this context remains a challenge.

#### Blastocyst and early post-implantation embryo stages

Acentriolar centrosomes are first observed at the 16- to 32-cell stage in the mouse embryo (Fig. 2A) (Gueth-Hallonet et al., 1993). Microtubule live tracking during the emergence of centrosomes has revealed that neither a radial microtubule organisation emanating from the *de novo* centrosomes nor a deviation from microtubule organisation mediated by the interphase bridges (Howe and FitzHarris, 2013). These findings support the idea that the centrosome is not yet fully functional during late pre-implantation development and that microtubule organisation is continuously controlled by the non-centrosomal interphase bridge (Zenker et al., 2017). Furthermore, γ-tubulin is expressed only at the spindle poles prior to the 32-cell stage in mice (Calarco-Gillam et al., 1983; Hiraoka et al., 1989). Subsequently, using immunogold electron microscopy, γ-tubulin has been detected around the two centrosomes in trophectoderm cells of fully expanded mouse blastocysts (Gueth-Hallonet et al., 1993). Thus, γ-tubulin might adopt roles for microtubule nucleation and centrosome maturation during the transition from pre- to post-implantation embryogenesis (Fig. 2A) (Gueth-Hallonet et al., 1993). However, when these structures become an active MTOC remains unknown (Gueth-Hallonet et al., 1993; Howe and FitzHarris, 2013).

Beginning at the blastocyst stage, centriole *de novo* formation requires SAS-4 (Xiao et al., 2021); mice lacking *Sas4* (*Cenpj*) cannot form centrioles *de novo*, impairing early post-implantation development before embryonic arrest around embryonic day 9 (Bazzi and Anderson, 2014; Xiao et al., 2021). This embryonic lethality might be caused by the gradual contribution of centrioles to mitosis and cilia formation around gastrulation (Bazzi and Anderson, 2014; Xiao et al., 2021). These findings suggest that centriole-containing centrosomes are not yet required during mammalian pre-implantation development; however, their maturation becomes essential post-implantation (Bazzi and Anderson, 2014; Xiao et al., 2021). Studies using live imaging in other species have provided further clues to why the co-existence of ncMTOCs and the centrosome might be pivotal as the embryo approaches gastrulation. For example, in addition to apical centrosome-derived microtubules, gastrulation in chicken embryos is enabled by a second non-centrosomal microtubule network (Nakaya et al., 2013). Similarly, in *D. melanogaster* embryos, a patronin-dependent (see CAMSAP3 in Glossary, Box 1) non-centrosomal microtubule network situated on top of the active centrosome is crucial for mesoderm invagination during gastrulation (Ko et al., 2019; Takeda et al., 2018). In addition, epithelial-like cells of the *D. melanogaster* gastrula exhibit a

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tyrosinated-to-acetylated microtubule gradient from apical to basal (Takeda et al., 2018), a feature also observed in the early stages of mouse pre-implantation embryos (Houliston and Maro, 1989). In *D. rerio*, two distinct microtubule arrays are present: inter-crossing interphase microtubules in the yolk syncytial layer; and a second array located in the yolk cytoplasmic layer, which extends in the direction of epibolic movement of the blastoderm (Fig. 2B) (Kimmel and Law, 1985; Solnica-Krezel and Driever, 1994). More recently, using total internal reflection fluorescence microscopy and fluorescently labelled microtubules in mutants of *pou5f3*, the *D. rerio* OCT4 homologue, have been shown to display slower velocity of microtubule dynamics due to the lack of microtubule regulation at the yolk cytoplasmic layer for epiboly (Eckerle et al., 2018). Collectively, these studies demonstrate the potential necessity of a dual microtubule network as the embryo exits naïve pluripotency and approaches gastrulation.

## The microtubule architecture of *in vitro* pluripotent stem cells

Since the first ESC lines were successfully established *in vitro* from the mouse blastocyst in 1981 (Evans and Kaufman, 1981; Martin, 1981), improved cell culture conditions and characterisation of PSCs have aided the derivation of ESCs from other species and the development of induced PSCs (Takahashi and Yamanaka, 2006). Correspondingly, defined cell culture conditions have enabled PSC lines to be maintained in the naïve, formative and primed pluripotency states (Kinoshita et al., 2021; Kinoshita and Smith, 2018; Wang et al., 2021). Despite these advances, our knowledge of the microtubule architecture in PSCs remains limited to a few studies, which is an insufficient reflection of their biological importance. A gene expression analysis of cytoskeletal markers in ESCs, induced PSCs and unprogrammed mouse embryonic fibroblasts has demonstrated no changes in tubulin α1b expression levels, despite a less complex cytoskeletal network in both ESCs and induced PSCs, compared with fibroblasts (Boraas et al., 2016). However, owing to the dynamic nature of the microtubule cytoskeleton, gene expression analyses may not capture the structural changes of microtubule organisation. In fact, advanced imaging of mouse naïve ESCs has revealed microtubule-dependent cytoplasmic bridges connecting sister cells (Chaigne et al., 2020), similar to the CAMSAP3-dependent interphase bridges in the pre-implantation mouse embryo (Fig. 2A; Fig. 3A,C). Remarkably, these microtubule bridges are retained throughout a prolonged period of the cell cycle, and their abscission results in naïve pluripotency exit (Fig. 4D). Furthermore, an exchange of cytoplasmic green fluorescent protein has been observed between the connected sister cells, indicative of roles in mediating intercellular communication. As mouse ESCs undergo controlled naïve pluripotency exit and transition to the subsequent pluripotency states, γ-tubulin has been shown to migrate to the apical membrane of the cells (Shahbazi et al., 2017). Presence of both γ-tubulin and the microtubule intercellular bridge in mouse PSCs raises the question about the microtubule nucleation potential of the active MTOC.

Undoubtedly, there are striking similarities between the microtubule bridge in naïve ESCs and the interphase bridge in the pre-implantation mouse embryo (Fig. 3A,B), possibly serving as the default ncMTOC in mammalian PSCs. In support of this idea, $Sas4^{-/-}$ and $Sas4^{+/+}$ mouse ESCs display identical pluripotency capacity, as demonstrated by NANOG expression (Xiao et al., 2021). Furthermore, γ-tubulin has been observed in $Sas4^{+/+}$ mouse ECSs but is absent from $Sas4^{-/-}$ cells. Despite the absence of γ-tubulin, $Sas4^{-/-}$ mouse ECSs remain viable; however, they undergo slower proliferation rates compared with $Sas4^{+/+}$ cells (Xiao et al., 2021). These findings suggest a switch between mitotic centrosome and ncMTOC activity during interphase (Xiao et al., 2021). Although this hypothesis requires further investigation, the current evidence highlights the importance of the unique organisation of microtubules in PSCs.

## Pluripotency and microtubule-dependent organelle dynamics

The formation of a distinct microtubule network is accompanied by the spatiotemporal reorganisation of organelles. The magnitude and significance of microtubule-dependent subcellular dynamics for embryo development and PSCs remain a challenging question.

Stereotypically, organelles are actively transported along microtubule filaments via motor protein-dependent mechanisms (Hirokawa et al., 2009; Sweeney and Holzbaur, 2018); however, intracellular transport can also occur via hitchhiking (Guo et al., 2018; Liao et al., 2019; Salogiannis and Reck-Peterson, 2017). Recently, tunnelling nanotubes have emerged as a mode of intercellular communication between PSCs undergoing differentiation (Resnik et al., 2018). These tunnels serve as a conduit for exchange of small molecules and organelles, such as mitochondria and lysosomes (Bukoreshtliev et al., 2009). Yet, whether other microtubule structures such as the intercellular bridges also facilitate transport between cells *in vivo* remains to be determined.

Over the past decades, mitochondrial bioenergetics have emerged as a hallmark for embryonic development and cellular reprogramming (Bahat and Gross, 2019), with mitochondrial number considered as a key selection criterion for embryo viability in reproductive medicine (Cecchino and Garcia-Velasco, 2019). However, increased scepticism has arisen due to the complexity of mitochondrial biology. A more comprehensive understanding of mitochondrial metabolism in relation to their dynamic activities, spatial real-time organisation and inter-organelle interactions is essential for their establishment as a robust and reliable biomarker. Spatiotemporal organelle-organelle dynamics orchestrated by microtubule post-translational modifications occur in various differentiated cell types (Koppers and Farías, 2021). For example, mitochondria-organelle contacts are preferentially located on acetylated microtubules (Friedman et al., 2011, 2010; Guo et al., 2018), while lysosome fusion occurs on tyrosinated microtubules (Burute and Kapitein, 2019). Such differences in organelle-microtubule associations can mediate disparities in the subcellular localisation of organelles and, thus, cell polarisation and asymmetries.

In the pre-implantation mouse embryo, glucose products act as key signalling factors for the segregation of outer cells from the ICM (Chi et al., 2020). It remains to be investigated whether these findings are linked to a preferential recruitment of mitochondria to the basal domain along acetylated microtubules at the late eight-cell stage for subsequent inheritance by inner cells at the 16-cell stage (Fig. 4B) (Ducibella et al., 1977; Houliston and Maro, 1989; Houliston et al., 1987, 1989; Zenker et al., 2017). Moreover, tyrosinated microtubules position endosomes towards a more-apical location at the late eight-cell stage (Fig. 4B) and lysosomes to the basal membrane towards the late 16-cell stage (Fig. 4C) (Fleming et al., 1986; Fleming and Pickering, 1985). Mouse blastocysts lacking cytoplasmic dynein, display fragmented and dispersed Golgi as well as dispersed endosomes and lysosomes throughout the cytoplasm, and then fail to implant (Harada et al.,

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1998). Similarly, disruption of microtubules following nocodazole treatment has shown that endosomes and lysosomes are dispersed throughout blastomeres of the mammalian pre-implantation embryo (Maro et al., 1985). Despite these seminal findings, there is a profound need to advance our understanding regarding the spatial real-time organisation of organelles regulating mammalian embryogenesis. Known to coordinate endosome trafficking (Khanal et al., 2016; Wong et al., 2018; Zenker et al., 2017), Golgi construction and positioning (Toya et al., 2016; Wang et al., 2017), as well as mitochondrial shape (Mitsuhata et al., 2021), CAMSAP3 has the ability to emerge as a master regulator for such processes.

Visualising the complex interplay of organelles in *D. rerio* embryos using a combination of lattice-light sheet microscopy and adaptive optics has identified how mitochondrial morphology and dynamics are coordinated *in vivo* (Liu et al., 2018). 3D imaging of whole *D. rerio* embryos has revealed the preferential localisation of a *trans*-Golgi network along the axis of polarisation, differences in endoplasmic reticulum distribution during interphase and more puncta-like mitochondria (Liu et al., 2018). At 14 h post fertilisation, the Golgi network and mitochondria are divided asymmetrically between daughter cells, resulting in notable differences in organelle size and morphology across developmental stages (Liu et al., 2018). The importance of mitochondrial dynamics has also been revealed in *C. elegans* zygotes using live imaging where the asymmetric distribution of mitochondria-derived hydrogen peroxide has been identified as a requirement for symmetry breaking (De Henau et al., 2020). Thus, microtubule arrangements may regulate the biased intracellular localisation of organelles to reinforce asymmetry and determine cell polarity.

The importance of organelle morphology and function for cell identity and pluripotency has also been demonstrated *in vitro*. In naïve PSCs, mitochondria are fragmented with undeveloped cristae, similar to pre-implantation embryos. Importantly, this characteristic can be observed even before the expression of pluripotency markers during cellular reprogramming (Prieto et al., 2020; Stadtfeld et al., 2008). As PSCs transition from a naïve to a primed state, mitochondria undergo fusion to elongate and mature, which is characterised by inner cristae (Nishimura et al., 2019; Zhong et al., 2019). In mouse ESCs lacking the mitochondrial carrier homolog MTCH2, an outer mitochondrial membrane protein, failure of mitochondrial fusion hinders subsequent conversion to a primed state (Bahat et al., 2018). In contrast, inducing mitochondrial elongation using pro-fusion protein Mitofusin (MFN2) or dynamin-related protein (DRP1) facilitated the exit from naïve pluripotency (Bahat et al., 2018). Thus, the hierarchy of structural events, including microtubule-driven mitochondrial dynamics and changes in gene expression during the generation of PSCs, needs to be assessed carefully. A comparison of human and mouse PSCs has demonstrated that basal oxygen consumption rate in primed PSCs is lower than in naïve PSCs (Takashima et al., 2014). These findings suggest structural mitochondrial changes may influence pluripotency beyond energy production and prior to genetic upregulation of transcription factors such as OCT4. More recently, a systems-level spectral imaging approach provided insights into the kinetic interactions between mitochondria and various organelles, including endoplasmic reticulum and lysosomes (Valm et al., 2017). These interactions synergistically drive mitochondrial remodelling events (Guo et al., 2018; Liu et al., 2018; Valm et al., 2017). Notably, grazing incidence structured illumination microscopy (GI-SIM) has demonstrated that the fusion of two mitochondria can be facilitated by the movement of lysosomes (Guo et al., 2018; Valm et al., 2017; Wong et al., 2018). Upon disruption of the microtubule network, organelle contacts are modulated, thus uncovering pivotal roles for the microtubule cytoskeleton in regulating inter-organelle interactions (Valm et al., 2017). Similarly, the microtubule interactor GTPase DRP1 has been identified as a key factor in coordinating endoplasmic reticulum-driven mitochondrial fission in collaboration with the *trans*-Golgi network (Nagashima et al., 2020; Tábara et al., 2021; Wong et al., 2018).

In addition to mitochondria, lysosomes are emerging as a pivotal regulator of pluripotency (Julian and Stanford, 2020). The lysosome-dependent process of autophagy regulates cellular homeostasis in ESCs and significantly influences the pluripotent status of these cells (Gu et al., 2019). Impairing autophagosome biogenesis in mouse induced PSCs negatively affects mitochondrial remodelling, leading to reduced self-renewal capability, reprogramming efficiency and pluripotency-associated protein expression (Liu et al., 2017). Furthermore, genetic analyses have demonstrated that the three key pluripotency-associated proteins OCT4, SOX2 and NANOG are maintained by high levels of autophagy (Liu et al., 2017). In human ESCs, the deletion of the microtubule-associated protein 1A/B light chain 3-I (LC3) causes an accumulation of OCT4, but a reduction of pluripotency (Cho et al., 2014). This finding demonstrates that autophagosome biogenesis, and thus pluripotency, is in part mediated by interactions with the microtubule cytoskeleton. It will be fascinating to observe how lysosomes might come to the forefront in stem cell and developmental research.

## Non-invasive manipulations: new approaches to alter subcellular organisation

Microtubule targeting agents are widely used in clinical therapies, e.g. for cancer and neurodegenerative diseases. However, microtubules are found in all eukaryotic cells. Consequently, microtubule targeting agents also act on ubiquitous microtubule-dependent mechanisms, such as cell division and transport, and thus cause severe adverse effects. The development of light-targeted techniques might offer enormous potential to non-invasively investigate and manipulate subcellular organisation with spatiotemporal precision (Kichuk et al., 2021). Modulation of the microtubule network by light to alter pronuclear migration dates back to the last century (Hamaguchi and Hiromata, 1986). Recently, advances in pharmacology and optogenetics have led to the generation of highly valuable microtubule-specific compounds, enabling reversible spatiotemporal control of microtubule organisation. Photostatins are photo-switchable microtubule growth inhibitors activated by visible light (Borowiak et al., 2015) and, when bound to tubulin, illuminated photostatins block microtubule growth in a sub-second response. Notably, paclitaxel analogues, which are microtubule stabilising compounds, are the newest engineered photo-switches (Müller-Deku et al., 2020). Furthermore, using an optogenetically controllable LOV2/Zdk1 cassette, microtubule growth can be attenuated with multi-scale precision by the photo-inactivable EB1 variant (van Haren et al., 2018). Although photo-pharmacology and optogenetics are *a priori* widely applicable, the use of such techniques is currently undervalued for the study of pluripotent cells. Innovative research using defined optogenetic stimulation of the Wnt signalling pathway on human ESCs has been successfully applied to induce self-organisation and embryonic patterning similar to human gastrulation (Repina et al., 2020). The challenge now is to develop such techniques further to alter the spatiotemporal dynamics of pluripotent cells, *in vivo* and at a subcellular level (Zenker et al., 2018, 2017). In addition, future microscope systems must enable the visualisation and identification of pairwise microtubule-organelle interactions at hyperspectral super-

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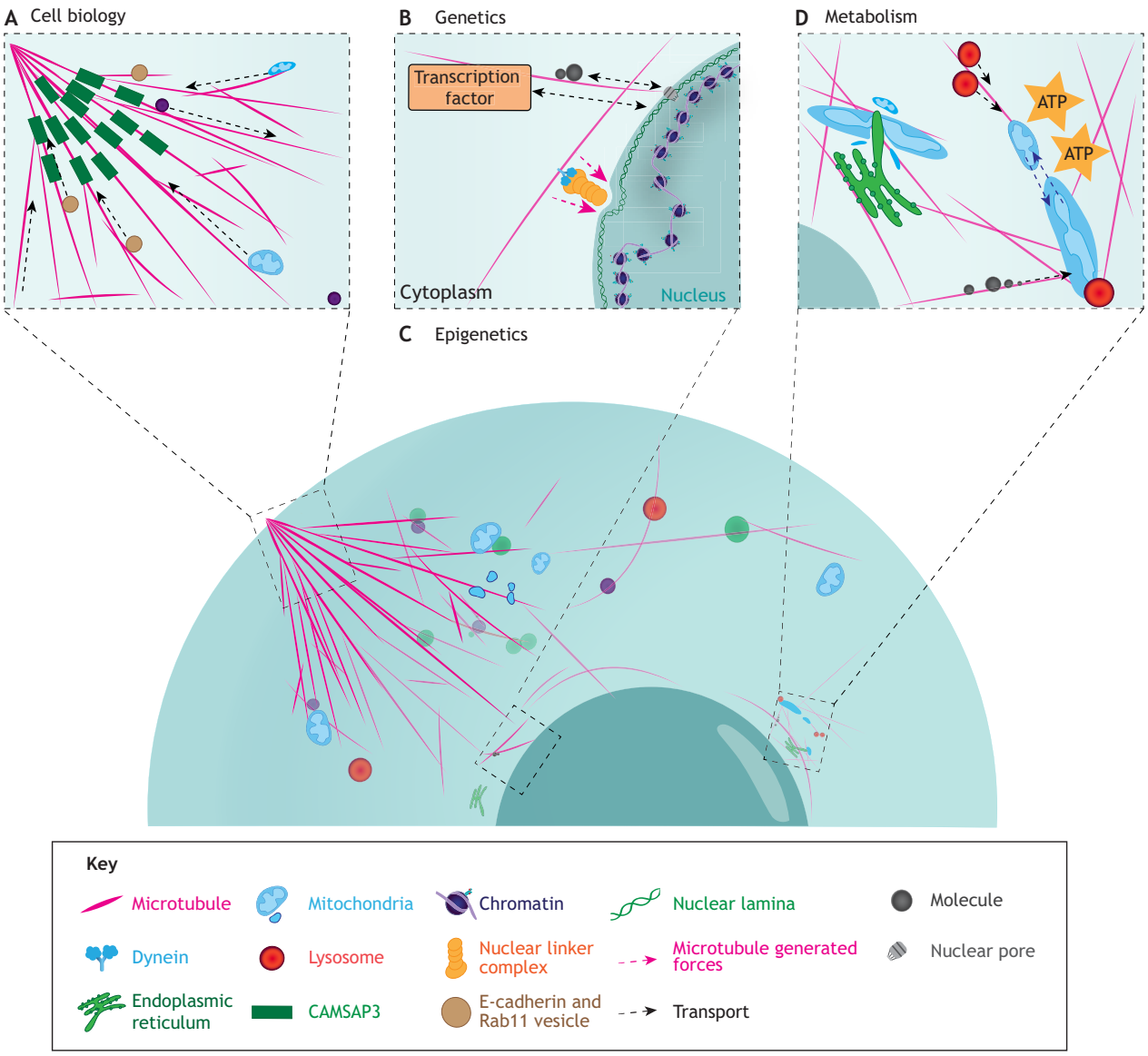


**Fig. 5. Orchestration of pluripotency identity by the microtubule cytoskeleton.** This schematic portrays how the microtubule cytoskeleton contributes to the establishment of pluripotent cell identity at all subcellular levels. (A) Cell biology: patterning of a pluripotent cell-specific microtubule organisation facilitating a unique subcellular architecture. (B) Genetics: a change in the transcriptional programme through microtubule-mediated cytoplasmic-nuclear shuttling of transcription factors and other molecules. (C) Epigenetics: a shift in chromatin organisation and genome stability through microtubule-generated forces on the nuclear envelope. (D) Metabolic: remodelling of mitochondrial dynamics, including ATP production, through microtubule-dependent organelle-organelle interactions. CAMSAP3, calmodulin-regulated spectrin-associated protein 3; ATP, adenosine triphosphate.

resolution. Spectral unmixing, in combination with advanced 2D techniques or GI-SIM, offers promising potential (Cutrale et al., 2017; Guo et al., 2018; Valm et al., 2017). Such techniques will need to ensure that whole tissues *in vivo* are optically accessible, such as novel non-invasive methods for 4D (3D plus time) imaging of mammalian embryos (Karnowski et al., 2017; McDole et al., 2018).

## Future perspectives

In this Review, we describe how the microtubule cytoskeleton coordinates processes essential for early embryo development and pluripotency, such as subcellular microtubule-dependent organelle dynamics. An integral part of pluripotency is the intrinsic self-organisation that determines cell fate. We propose that subcellular architecture configured by the microtubule cytoskeleton has a pivotal role in regulating pluripotency at genetic, epigenetic and metabolic levels (Fig. 5). Apparent links exist between microtubules, organelles and cell metabolism. This might be complemented soon by increasing evidence on how the microtubule cytoskeleton modulates gene expression (Shokrollahi and Mekhail, 2021), including zygotic genome activation (Hampoelz et al., 2019). Microtubules exert physical forces on the nucleus to trigger genomic reorganisation and serve as a non-genetic mode of determining nuclear architecture during interphase (Bustin and Misteli, 2016). Moreover, the direct connection between cytoplasmic microtubules and the nuclear envelope may facilitate the exchange of molecules and transcription factors (Maizels and Gerlitz, 2015; Sun et al., 2019; Zheng et al., 2020), or MTOCs may indirectly alter heterochromatin for subsequent alteration of gene expression (Gerlitz et al., 2013). Besides the

interaction with subcellular compartments, another way in which the microtubule network can influence pluripotency is through its inherent lattice plasticity. Modulated by various factors, such as microtubule associated proteins, post-translational modifications, motor proteins and mechanical strain, the microtubule lattice is an allosteric collective that adapts to its environment and cellular function (Cross, 2019). Looking forward, we anticipate that the combination of live imaging and optogenetics methods will transform our ability to specifically manipulate the microtubule network in order to trigger cell fate changes. These advances offer promising applications for deciphering the structural characteristics of pluripotency and the ability to identify the prospective connection between microtubule-dependent subcellular dynamics and cell potency.

Dissecting how intrinsic cellular regulation contributes to pluripotency might lead a revolutionary era of regenerative medicine. This exciting field has the potential to further advance regenerative and assisted reproductive medicine, including applications for safer and more efficient therapeutic use of induced PSCs, for the survival rate of *in vitro* fertilised embryos and for the early detection of cellular abnormalities *in vivo* and *in vitro*. Furthermore, the emerging field of *in vitro* generated self-organised embryo-like structures, such as embryoids, blastoids and gastruloids, requires simultaneous advances in live imaging techniques to evaluate their replicative potential (Liu et al., 2021; Yu et al., 2021). These methods may enable the direct comparison and validation of *in vitro* models to their *in vivo* counterparts. We propose that cell biology is the missing part of the pluripotency puzzle (Fig. 5) allowing its completion and framing through novel therapeutic applications.

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