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Use of *Xenopus* cell-free extracts to study size regulation of subcellular structures

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ABSTRACT Striking size variations are prominent throughout biology, at the organismal, cellular, and subcellular levels. Important fundamental questions concern organelle size regulation and how organelle size is regulated relative to cell size, also known as scaling. Uncovering mechanisms of organelle size regulation will inform the functional significance of size as well as the implications of misregulated size, for instance in the case of nuclear enlargement in cancer. *Xenopus* egg and embryo extracts are powerful cell-free systems that have been utilized extensively for mechanistic and functional studies of various organelles and subcellular structures. The open biochemical nature of the extract permits facile manipulation of its composition, and in recent years extract approaches have illuminated mechanisms of organelle size regulation. This review largely focuses on *in vitro Xenopus* studies that have identified regulators of nuclear and spindle size. We also discuss potential relationships between size scaling of the nucleus and spindle, size regulation of other subcellular structures, and extract experiments that have clarified developmental timing mechanisms. We conclude by offering some future prospects, notably the integration of *Xenopus* extract with microfluidic technology.

KEY WORDS: *nuclear size, spindle size, developmental size scaling, midblastula transition, cancer, microfluidics*

Introduction

Size is an important defining feature in biology. Tremendous size variation exists at the species level, as well as at the organ level where organ size tends to correlate with organismal size. Cell size also varies among different eukaryotic species and cell types, and dramatic changes in cell size occur during development, cell division, and differentiation. While our understanding of how cell sizes are regulated is fairly advanced (Fingar *et al.*, 2002, Kozma and Thomas, 2002, Nurse, 1975), less is known about the mechanisms and functional significance of organelle size regulation.

Organelles adopt characteristic architectures, sizes, and shapes. How cells sense and maintain organelle size in order to ensure proper function is an important and largely unexplored question. A related intriguing question is how the sizes and/or numbers of organelles and other subcellular structures are regulated relative to cell size. The sizes of many organelles, including the nucleus and spindle, positively correlate with cell size; large cells tend to have proportionally larger organelles, a phenomenon referred to as scaling (Edens and Levy, 2014b, Jevtic and Levy, 2015, Levy and Heald, 2012, Marshall, 2002, Mitchison *et al.*, 2015, Wilson, 1925). One model to explain organelle scaling invokes limiting pools of cytoplasmic components, where limiting structural proteins or enzymatic activities might restrict organelle size and/or number (Goehring and Hyman, 2012, Good *et al.*, 2013, Hazel *et al.*, 2013). Another possibility is that steady state organelle size is determined by a balance of organelle assembly and disassembly rates, which can also be affected by component limitation (Edens and Levy, 2014a, Loughlin *et al.*, 2011, Marshall *et al.*, 2005, Rafelski *et al.*, 2012, Reber *et al.*, 2013). In a related model, feedback based on organelle size or functional output might impact organelle growth (Marshall, 2012). Changes in organelle sizes and scaling relationships likely affect cell physiology, and

Abbreviations used in this paper: ER, endoplasmic reticulum; INM, inner nuclear membrane; MBT, midblastula transition; MT, microtubule; MTOC, microtubule organizing center; NE, nuclear envelope; NLS, nuclear localization signal; NPC, nuclear pore complex; ONM, outer nuclear membrane; SAF, spindle assembly factor.

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aberrations in organelle morphology are associated with certain disease states (Chow *et al.*, 2012, Jevtic and Levy, 2014, Zink *et al.*, 2004).

The *Xenopus* cell-free extract system has been utilized extensively to study different cellular processes including nuclear assembly and import (Chan and Forbes, 2006), chromosome structure (Maresca and Heald, 2006), and mitotic spindle regulation (Hannak and Heald, 2006). More recently, this *in vitro* system has provided a useful platform to study mechanisms of organelle size regulation. In this review we largely focus on *in vitro Xenopus* studies that have revealed regulators of nuclear and spindle size (Fig. 1).

Advantages of using *Xenopus* cell-free extracts to study organelle size regulation

Xenopus egg extract is a powerful cell-free system to study the mechanisms and functional significance of organelle size regulation. Detailed methods for the preparation of both *X. laevis* and *X. tropicalis* egg extracts have been described (Brown *et al.*, 2007, Desai *et al.*, 1999, Edens and Levy, 2014b, Hannak and Heald, 2006, Murray, 1991). Female *Xenopus* frogs are primed and induced to lay eggs with gonadotropin hormone injections. The egg jelly coats are removed, and washed eggs are subjected to a crushing centrifugation step, followed by collection of the nearly undiluted cytoplasm that contains the membranes and cytoplasmic proteins necessary to assemble organelles in vitro. Egg extracts do not contain the egg chromosomes, so addition of an exogenous chromatin source, usually demembranated *Xenopus* sperm chromatin, is necessary to initiate nuclear and spindle assembly. Laid amphibian eggs are arrested in metaphase of meiosis II, and simple manipulations allow for cycling of egg extracts between interphase and mitosis. Due to the open biochemical nature of the extract, recombinant proteins can be added or endogenous proteins can be immunodepleted or inhibited with neutralizing antibodies or small molecules. Addition of fluorescently labeled proteins or dyes allows for visualization of organelles and subcellular structures, performance of functional assays, and live time-lapse imaging of dynamic processes.

Similar to egg extracts, embryo extracts can be generated from both *X. laevis* and *X. tropicalis* embryos at different developmental stages. During early *Xenopus* embryogenesis, cell divisions are rapid without changes in the size of the embryo itself, and as such it provides a powerful cellular scaling system. In *X. laevis* the ~1.2 mm diameter fertilized egg undergoes twelve rapid, synchronous cell cycles (each approximately 25 to 30 minutes), to produce about four thousand 40-190 μm cells (average 100 μm diameter) at the midblastula transition (MBT), also referred to as stage 8.5 (Jevtic and Levy, 2015, Nieuwkoop and Faber, 1967). As the embryo proceeds through gastrulation (stages 10.5-12), further reductions in cell size occur, reaching 12-30 μm in the tadpole (average 20 μm diameter, our unpublished measurements). Both nuclear size and spindle size decrease throughout *Xenopus* early embryonic development, providing a robust system to investigate organelle scaling (Jevtic and Levy, 2015, Levy and Heald, 2010, Wuhr *et al.*, 2008).

The preparation and use of embryo extracts from different developmental stages are nearly the same as for egg extract (Edens and Levy, 2014b, Edens and Levy, 2016, Jevtic *et al.*, 2015). One notable difference is that endogenous organelles, such as nuclei and spindles, can be observed in their native cytoplasm, as it is not necessary to add an exogenous chromatin source to embryo extracts. Methods for manipulating extract composition and visualizing structures and activities are the same. Changes in scaling factor abundance and localization during development can be determined by immunoblotting different stage embryo extracts and performing immunofluorescence on embryos. The analysis and interpretation of embryo extract experiments is facilitated by recent transcriptomic and proteomic studies in *Xenopus* (Collart *et al.*, 2014, Peshkin *et al.*, 2015, Wuhr *et al.*, 2014, Yanai *et al.*, 2011). Thus, both egg and embryo extracts represent robust *in vitro* systems to elucidate mechanisms of organelle size regulation.

Nuclear structure and significance of nuclear size regulation

Many structural components of the nucleus contribute to the proper regulation of nuclear morphology. The nuclear envelope (NE) consists of an outer nuclear membrane (ONM) and inner nuclear membrane (INM), with the ONM being continuous with the endoplasmic reticulum (ER). Inserted at sites of ONM-INM fusion are nuclear pore complexes (NPCs) that mediate nucleocytoplasmic transport (Fig. 1). The classical nuclear import pathway is mediated by importin α, a nuclear import receptor that binds to cargos containing a nuclear localization signal (NLS), and importin β, that mediates interactions with the NPC. Inside the nucleus, Ran-GTP binds to importin β causing release of importin α and NLS cargos. Importin β bound to Ran-GTP is exported to the cytoplasm where nucleotide hydrolysis converts Ran-GTP to Ran-GDP. Nuclear transport factor 2 (NTF2) binds to both Ran-GDP and FxFG repeat-containing nucleoporins, acting as a dedicated nuclear import factor for Ran-GDP, which is converted to Ran-GTP in the nucleus by its guanine nucleotide exchange factor, chromatin-bound RCC1 (Fig. 1) (Madrid and Weis, 2006, Stewart, 2007). The INM is lined on its nucleoplasmic face by the nuclear lamina and chromatin. The nuclear lamina is a meshwork of intermediate filaments that provides mechanical strength to the nucleus and contributes to chromatin organization, transcriptional regulation, and DNA metabolism (Gruenbaum *et al.*, 2005, Walters *et al.*, 2012, Worman and Courvalin, 2005). The main structural components of the nuclear lamina are the nuclear lamin proteins, which in somatic vertebrate cells include lamin B1, B2, and A/C (Fig. 1) (Misteli and Spector, 2011, Parnaik *et al.*, 2011).

Changes in nuclear size are linked to normal physiological events such as development, cell division, and differentiation (Edens *et al.*, 2013, Jevtic *et al.*, 2014, Levy and Heald, 2012, Vukovic *et al.*, 2016a), while aberrations in nuclear size and shape are associated with aging (Prokocimer *et al.*, 2009) and cancer (Chow *et al.*, 2012, Jevtic and Levy, 2014). Enlarged nuclear size is a morphometric feature commonly used by cytopathologists to diagnose, stage, and prognose many different cancers (Zink *et al.*, 2004). The functional link between malignancy and altered nuclear size is not clear, and notably, cancer-associated changes in nuclear size often do not correlate with increased DNA content (Jevtic and Levy, 2014). Do changes in chromatin organization and gene expression during tumorigenesis result from or cause increased nuclear size? Using the *Xenopus* system to elucidate mechanisms of nuclear size regulation will provide insight into

the functional significance of nuclear size and improve our understanding of the link between altered nuclear size and disease states such as cancer.

Mechanisms of interspecies nuclear scaling

X. laevis frogs, cells, eggs, and nuclei are larger than those of *X. tropicalis*, offering a physiological scenario in which to investigate mechanisms of nuclear size control. Interspecies nuclear scaling was recapitulated using egg extracts from these two related *Xenopus* species. Nuclei assembled with *X. laevis* sperm chromatin in *X. laevis* egg cytoplasm expanded more rapidly and were larger than in *X. tropicalis* cytoplasm. Mixing the two different egg extracts produced nuclei of intermediate size, suggesting that titratable cytoplasmic factors regulate nuclear size in this system. Nuclei assembled with *X. tropicalis* sperm, having around half the DNA content of *X. laevis* sperm, were minimally smaller, showing that cytoplasm has a greater effect on nuclear size than bulk DNA content. Rates of nuclear import were found to differ between the two extracts, with faster import into *X. laevis* nuclei than *X. tropicalis* nuclei. The levels of two nuclear import factors were found to be different between the two extracts. *X. laevis* extract has a higher concentration of importin α, while *X. tropicalis* extract has a greater concentration of NTF2 (Fig. 2A). Altering the levels of both import factors was nearly sufficient to account for nuclear import and nuclear size differences between these two species (Levy and Heald, 2010). Both factors modulate lamin B3 (LB3) import, the major egg lamin required for NE growth (Jenkins *et al.*, 1993, Newport *et al.*, 1990), with importin α increasing overall import rates and NTF2 reducing import based on cargo size (Levy and Heald, 2010). These experiments demonstrated a role for nuclear import and nuclear structural components as physiological regulators of nuclear size in *Xenopus.*

As a follow-up to these studies, we tested how NTF2 concentrations and mutants affect nuclear size in *X. laevis* egg and embryo extracts. Using a mutant version of NTF2 that is defective for Ran binding, we showed that the ability of NTF2 to inhibit nuclear growth and import of large cargo molecules, such as lamins, depends on its binding to Ran. An NTF2 mutant with reduced affinity for the NPC was also defective in limiting nuclear growth, thus implicating binding of NTF2 to both Ran and the NPC in nuclear size regulation. Ectopic NTF2 expression in *Xenopus* early embryos also

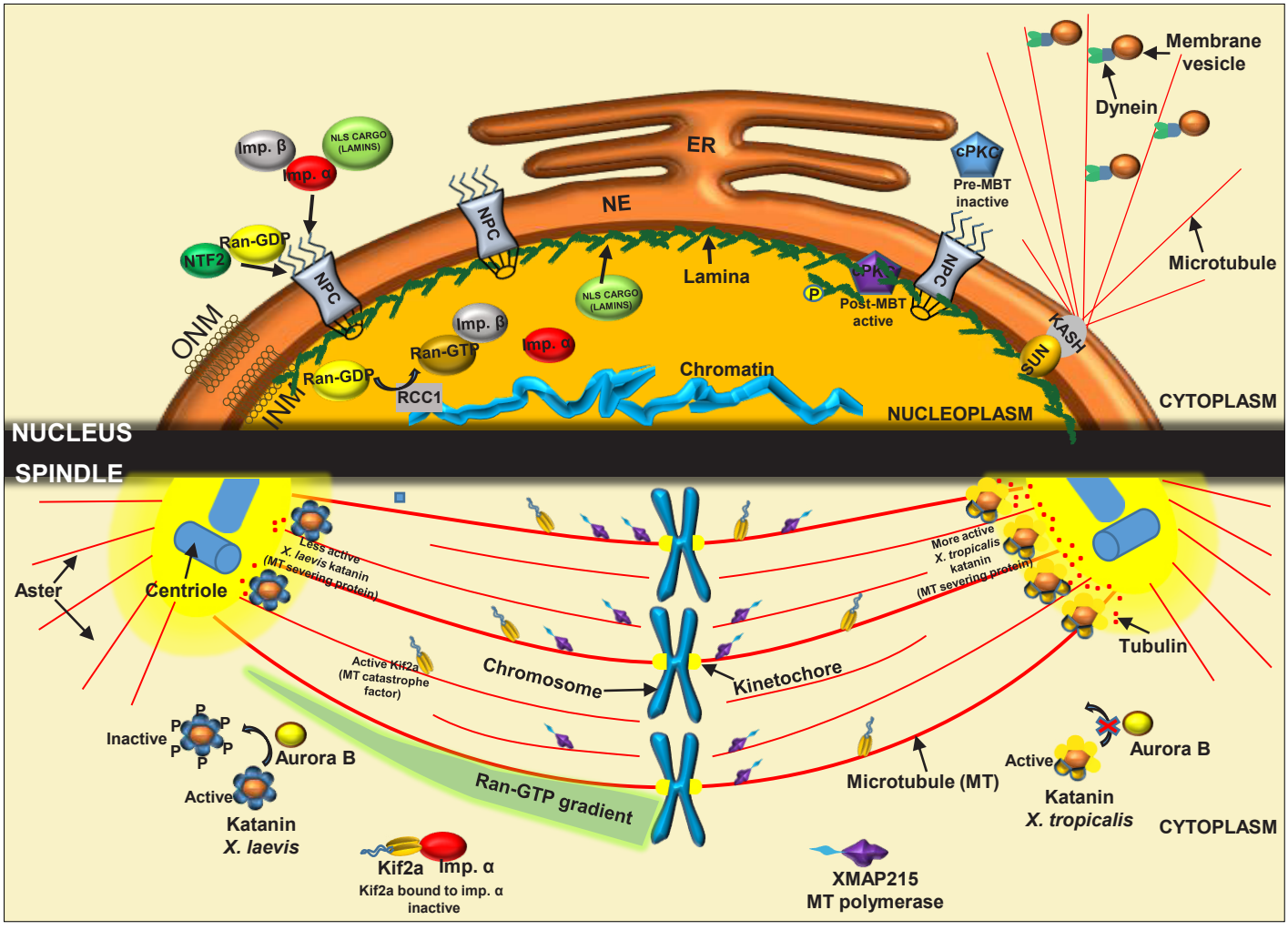

Fig. 1. Summary of some of the mechanisms that contribute to the regulation of nuclear and spindle size that were elucidated using *Xenopus* extracts.

led to altered nuclear size. We propose that NTF2 bound to Ran reduces the effective diameter of the NPC pore, inhibiting import of large cargos and nuclear expansion. In support of this model, transmission electron microscopy showed that egg extract nuclei supplemented with wild-type NTF2 exhibited a 20-30% reduction in average NPC diameter (Fig. 2B). In contrast, the Ran binding mutant, though still effectively recruited to the NPC, did not alter NPC size (Vukovic *et al.*, 2016b). This new mechanistic information about how NTF2 affects nuclear size will facilitate future studies into the functional significance of nuclear size during development, differentiation, and carcinogenesis.

Mechanisms of developmental nuclear scaling

During *X. laevis* early embryogenesis, nuclear volume decreases ~3-fold during the cleavage cell divisions and up to the MBT, and this reduction in nuclear size correlates with reductions in cytoplasmic importin α levels and bulk import (Levy and Heald, 2010, Wilbur and Heald, 2013). From the MBT through gastrulation, another 3-fold reduction in nuclear volume occurs. To test what contributes to this post-MBT nuclear size scaling, we developed an *in vitro* nuclear re-sizing assay using *Xenopus* embryo extracts. When large egg extract nuclei were incubated in gastrula stage embryonic cytoplasm, the nuclei became smaller. Further experiments showed that this nuclear shrinking activity present in late stage embryonic cytoplasm was dependent on conventional protein kinase C (cPKC). Both cPKC activity and nuclear localization were observed to increase in post-MBT embryos, correlating with reduced nuclear size and decreased nuclear localization of lamins (Fig. 1, Fig. 2C). Furthermore, manipulating cPKC activity *in vivo* was sufficient to alter interphase nuclear size in post-MBT embryos. From these studies, we propose that steady-state nuclear size is established through a balance of nuclear import-mediated growth and cPKC-dependent nuclear shrinking (Edens and Levy, 2014a). Future studies will address if nuclear lamins are directly phosphorylated by cPKC to regulate interphase lamina dynamics and nuclear size. How these developmental changes in nuclear size and dynamics affect nuclear mechanics is a question for future study.

We further investigated how nuclear lamins affect nuclear size in *X. laevis* egg and embryo extracts. During early *X. laevis* development, the expression of different lamin isoforms changes, as does the total lamin concentration (Benavente *et al.*, 1985, Jevtic *et al.*, 2015, Stick and Hausen, 1985). To test how the level and type of lamin expression influence nuclear size, we titrated recombinant lamin proteins into egg and embryo extracts. We found that nuclear growth and size were sensitive to the levels of nuclear lamins, with low and high concentrations increasing and decreasing nuclear size, respectively (Fig. 2D). Interestingly, each type of lamin we tested (lamin B1, B2, B3, A) similarly affected nuclear size, whether added alone or in combination. These data suggest that total lamin concentration, and not lamin type, is more critical in determining nuclear size (Jevtic *et al.*, 2015). How might relatively low levels of ectopic lamins increase nuclear size? Nuclear import capacity in *X. laevis* egg extract is extremely high, so even relatively low amounts of added lamins might be expected to alter nuclear size. Why might higher lamin levels reduce nuclear size? It is possible that when lamins are imported into the nucleus too rapidly, due to fast import kinetics, they are unable to properly incorporate into the lamina, leading to observed intranuclear lamin aggregates. These aggregates may have a dominant-negative effect, extracting endogenous lamins from the nuclear lamina and leading to a reduction in nuclear size. In support of this idea, high concentrations of a LB3 CAAX box mutant, defective for farnesylation and targeting to the lamina, still formed intranuclear aggregates and decreased nuclear size, while

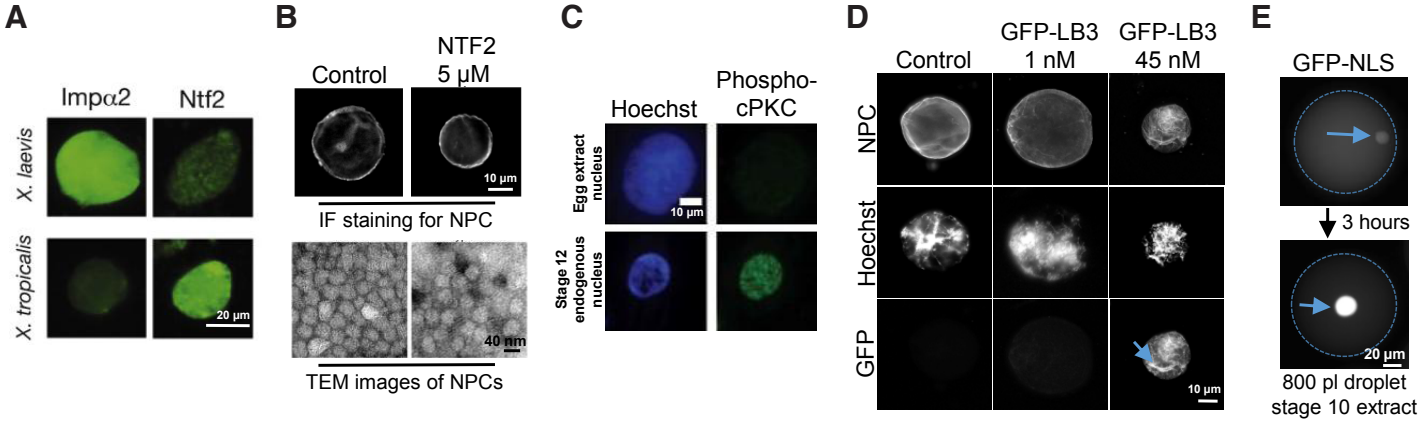

Fig. 2. Examples of nuclear size regulatory mechanisms in *Xenopus*. (A) *Nuclei assembled in* X. laevis *egg extract are larger than nuclei in* X. tropicalis *extract, correlating with higher nuclear importin α2 levels and lower nuclear NTF2 levels in* X. laevis. *Image adapted with permission from (Levy and Heald, 2010).* **(B)** *Supplementing* X. laevis *egg extract nuclei with recombinant NTF2 reduces nuclear size, correlating with a reduction in NPC diameter as determined by transmission electron microscopy (TEM). This research was originally published in (Vukovic* et al., *2016b).* **(C)** *Compared to large nuclei that assemble in* X. laevis *egg extract and early embryos, nuclei are smaller in later stages of development. This developmental scaling of nuclear size correlates with an increase in nuclear cPKC localization and activity. This research was originally published in (Edens and Levy, 2014a).* **(D)** X. laevis *egg extract nuclei supplemented with low or high concentrations of GFP-lamin B3 (GFP-LB3) are larger or smaller than controls, respectively. Small nuclear size correlates with the formation of intranuclear lamin aggregates, indicated by the arrow. This research was originally published in (Jevtic* et al., *2015).* **(E)** *Stage 10* X. laevis *embryonic cytoplasm and endogenous nuclei were encapsulated in large 800 pl droplets using a microfluidic device. Nuclei are visualized by import of GFP-NLS. The droplet periphery is indicated with dotted blue lines, and nuclei are indicated with arrows. Nuclear size expands over the course of ~3 hours at room temperature (our observation).*

high concentrations of a LB3 point mutant defective for lamina assembly did not (Jevtic *et al.*, 2015).

Other factors that influence nuclear size

A number of additional factors that impact nuclear size have been studied using *Xenopus* extracts. Depletion of the nucleoporin Nup188 from *Xenopus* egg extract caused accelerated import of INM proteins with a concomitant increase in nuclear size (Theerthagiri *et al.*, 2010). Addition of a dominant-negative fragment of POM121, an integral membrane nucleoporin, to egg extract inhibited NE growth (Shaulov *et al.*, 2011). TPX2, an important regulator of spindle assembly and size (Helmke and Heald, 2014), localizes to the nucleus in interphase by binding lamina-associated polypeptide 2 (LAP2). Depletion of TPX2 from *Xenopus* egg extract led to the formation of small nuclei (O'Brien and Wiese, 2006), and addition of the chromatin- or lamin-binding domain of LAP2 to *Xenopus* egg extract inhibited nuclear growth (Gant *et al.*, 1999). These data suggest that TPX2 and its interaction with LAP2 are required for normal nuclear size regulation, and that lamina-chromatin interactions contribute to proper nuclear size determination.

Extranuclear structures also contribute to the regulation of nuclear size and morphology. During late mitosis, clearance of microtubules (MTs) from the recently segregated chromosomes is important for normal nuclear assembly and morphology in the subsequent interphase. One protein responsible for removing these MTs is the developmental pluripotency-associated 2 (Dppa2) protein that binds to chromatin and inhibits MT polymerization. Depletion of Dppa2 from *Xenopus* egg extract led to inappropriate accumulation of MTs around the post-mitotic chromatin, causing the formation of small misshapen nuclei. Strikingly, treatment with MT depolymerizing drugs rescued this nuclear morphology defect (Xue *et al.*, 2013). Clearance of membrane from mitotic chromosomes is also important for normal nuclear morphology. A biochemical screen for MT-membrane linkers using *Xenopus* egg extract identified REEP4, and depletion of the MT-binding ER proteins REEP3/4 in HeLa cells resulted in inappropriate membrane accumulation around metaphase chromosomes with concomitant NE defects during interphase (Schlaitz *et al.*, 2013).

Also important in nuclear size regulation is the ER, an interconnected membrane network consisting of highly curved ER tubules and flat cisternal sheets. The NE is continuous with the ER, so alterations in ER composition can affect nuclear morphology. Experiments using fractionated *Xenopus* egg extract showed that NE assembly involves targeting of ER tubules to chromatin, followed by membrane spreading around the chromatin. Disruption of ER membranes using shear mechanical stress inhibited nuclear growth, and reformation of an ER network allowed for NE re-growth (Anderson and Hetzer, 2007). Proteins in the reticulon (Rtn) and REEP families shape ER tubules by inserting hydrophobic wedges into the lipid bilayer to induce membrane curvature (Voeltz *et al.*, 2006, West *et al.*, 2011). Nuclei failed to form in egg extract treated with an Rtn4a neutralizing antibody, and disruption of ER network re-formation using an inhibitory antibody against Ufd1 (an adapter protein of the AAA-ATPase p97 required for ER network maintenance) slowed the growth of pre-assembled nuclei (Anderson and Hetzer, 2007). These results demonstrate that a tubular ER is necessary for both nuclear assembly and expansion. Rtn4 overexpression in early *Xenopus* embryos also altered nuclear size (Jevtic and Levy, 2015). Rtns are thought to impact nuclear size through a tug-of-war competition between nuclear and ER membrane availability, such that an increase in ER tubulation leads to a reduction in NE membrane and nuclear size (Anderson and Hetzer, 2008). Important questions for future research are how cytoplasmic volume affects ER size and morphology, and how such ER scaling might impact the regulation of nuclear size.

Contributions of nuclear volume, cytoplasmic volume, and DNA amount to MBT timing

The MBT is the first major developmental transition during early *X. laevis* embryogenesis when abrupt zygotic transcription begins and cell cycles elongate. Elegant experiments in which cytoplasmic volume or DNA amount were varied in embryos supported the idea that the DNA-to-cytoplasmic volume ratio regulates MBT onset. By this model, maternally derived DNA-binding MBT inhibitors present in a fixed cytoplasmic volume are titrated against exponentially increasing genomic DNA amounts during the cleavage stages of early development. Once a critical DNA amount is reached that exceeds the binding capacity of available inhibitors, the MBT is induced (Clute and Masui, 1995, Newport and Kirschner, 1982a, Newport and Kirschner, 1982b). Recently, a number of potential limiting components have been identified as candidate regulators of MBT onset. The levels of four DNA replication initiation factors (Cut5, RecQ4, Treslin, and Drf1) were fully titrated from egg extract cytoplasm at a DNA-to-cytoplasm ratio characteristic of the MBT. Overexpression of all four DNA replication factors in *Xenopus* embryos increased DNA replication rates by increasing origin firing and delayed the MBT as evidenced by a delay in the onset of zygotic gene expression and greater numbers of rapid synchronous cell divisions prior to cell cycle lengthening (Collart *et al.*, 2013). Another *Xenopus* extract study also found that DNA replication rates and origin firing are reduced at the high DNA-to-cytoplasm ratios found at the MBT. However, in this study, the limiting components were identified as the protein phosphatase PP2A and its regulatory subunit B55α (Murphy and Michael, 2013).

X. laevis egg extract has also been used to identify factors that directly regulate the onset of zygotic gene expression at the MBT. By adding increasing amounts of sperm chromatin to a fixed volume of egg extract, a critical DNA-to-cytoplasm ratio, similar to that found in the embryo at the MBT, was capable of triggering new transcription. Removal of DNA-binding proteins from extract using DNA-coated magnetic beads generated an extract capable of activating transcription at much lower concentrations of sperm chromatin, demonstrating that the transcriptional inhibitor is a DNA-binding protein. Subsequent biochemical fractionation identified histones H3 and H4 as the putative MBT inhibitors. Supplementing egg extract with recombinant H3 and H4 increased the DNA threshold required for activation of transcription in a dosage-dependent manner, while histone depletion allowed for transcriptional activation with lower DNA amounts. In addition, manipulating histone levels *in vivo* affected the timing of MBT-associated changes in cell cycle lengths (Amodeo *et al.*, 2015).

The DNA-to-cytoplasm ratio may not be the only parameter that determines MBT timing. During early *X. laevis* embryogenesis, cell size scales smaller at a much faster rate than nuclear size. From stage 4 to stage 8.5, average nuclear volume reduces

~3-fold, while cytoplasmic volume shows a much more dramatic ~70-fold reduction. As a consequence of this difference in scaling rates, the nuclear-to-cytoplasmic (N/C) volume ratio increases rapidly during early development, reaching a maximum at the MBT (Jevtic and Levy, 2015). To test how nuclear size and the N/C volume ratio impact the onset of the MBT, we altered nuclear size in *X. laevis* embryos by ectopically expressing nuclear scaling factors known to affect nuclear size: importins, lamins, and reticulons. We found that increasing nuclear size and the N/C volume ratio led to premature onset of zygotic gene transcription and cell cycle lengthening. Conversely, decreasing nuclear size in early embryos delayed activation of zygotic transcription and cell cycle lengthening (Jevtic and Levy, 2015). These studies raise a number of questions about the regulation of MBT timing. What are the relative contributions of nuclear size and DNA amount to MBT timing? Do changes in nuclear volume alter the intranuclear concentrations of limiting, maternally derived DNA binding factors? If so, the MBT might be regulated not only by the total amount of these maternally deposited limiting components but by their nuclear concentrations, as determined by changes in total nuclear volume during early embryogenesis. Do changes in nuclear size affect the MBT by altering chromatin compaction and organization? How might changes in NE surface area affect import capacity and the import of limiting DNA binding components that in turn regulate MBT timing? Future experiments using *Xenopus* egg and embryo extracts promise to provide answers.

Metaphase spindle structure and significance of spindle size regulation

Xenopus is an incredibly powerful model system for studying size regulation of the interphase nucleus, and it has been just as useful for elucidating mechanisms that control the size of the mitotic spindle. Like the nucleus, the size of the mitotic spindle scales with cell size during development (Courtois *et al.*, 2012, Good *et al.*, 2013, Wuhr *et al.*, 2008). The spindle apparatus plays a major role in cell division, ensuring proper chromosome segregation. While the length of the spindle seems to be important for accurate segregation of chromosomes (Schubert and Oud, 1997), it is unknown whether there is a causal link between altered spindle size and disease. The spindle is composed of dynamic microtubule (MT) arrays organized into a steady-state structure with two focused poles (Fig. 1). Approximately 150 well-characterized spindle assembly proteins regulate MT nucleation, polymerization dynamics, cross-linking, sliding, and focusing, regulated by kinases, phosphatases, and checkpoints (Sauer *et al.*, 2005). Spindle length, defined as the distance from one spindle pole to another, is tightly regulated during metaphase so that the distance between segregated anaphase chromosomes is sufficient to ensure each daughter cell receives a full complement of chromosomes [for review see (Goshima and Scholey, 2010)]. Since the number of spindle poles often dictates the number of daughter cells, accurate segregation of the duplicated genome and viability of daughter cells require a bipolar structure.

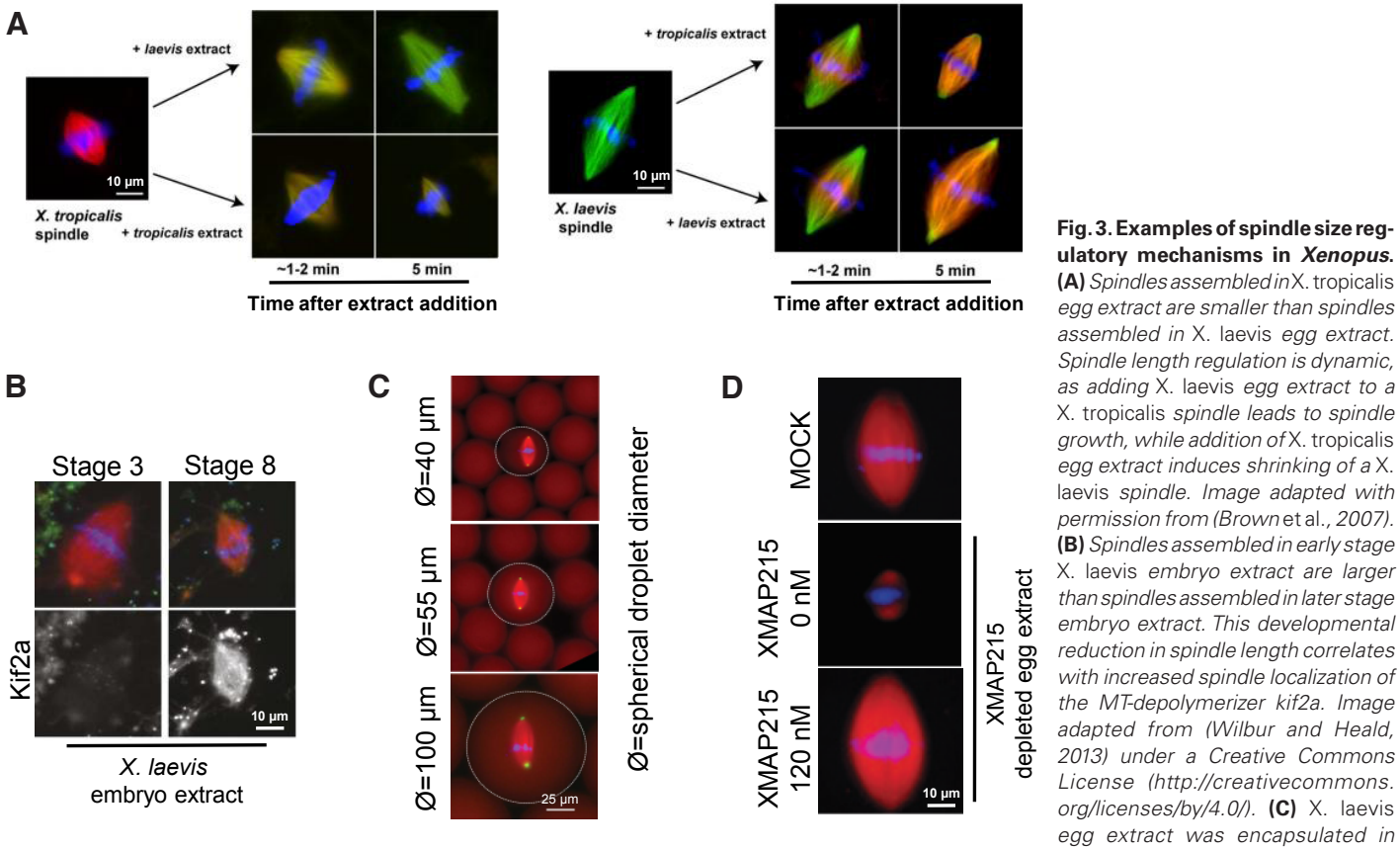

Fig. 3. Examples of spindle size regulatory mechanisms in *Xenopus*. **(A)** *Spindles assembled in* X. tropicalis *egg extract are smaller than spindles assembled in* X. laevis *egg extract. Spindle length regulation is dynamic, as adding* X. laevis *egg extract to a* X. tropicalis *spindle leads to spindle growth, while addition of* X. tropicalis *egg extract induces shrinking of a* X. laevis *spindle. Image adapted with permission from (Brown* et al.*, 2007).* **(B)** *Spindles assembled in early stage* X. laevis *embryo extract are larger than spindles assembled in later stage embryo extract. This developmental reduction in spindle length correlates with increased spindle localization of the MT-depolymerizer kif2a. Image adapted from (Wilbur and Heald, 2013) under a Creative Commons License (http://creativecommons.org/licenses/by/4.0/).* **(C)** X. laevis *egg extract was encapsulated in droplets of varying size using microfluidic devices. The length of the assembled spindle correlates with the volume of the droplet. Image adapted with permission from (Hazel* et al.*, 2013).* **(D)** *Spindles assembled in* X. laevis *egg extract depleted of the MT polymerase XMAP215 are small, and titration of recombinant XMAP215 into this extract results in a graded increase in spindle length. Image adapted with permission from (Reber* et al.*, 2013).*

This is particularly relevant in cancer cells containing supernumerary centrosomes (more than two) (Ganem *et al.*, 2009, Milunovic-Jevtic *et al.*, 2016). In the next few sections of this review, we will focus on how *Xenopus* egg and embryo extracts have provided insights into the mechanisms that control metaphase spindle size and shape.

Mechanisms of interspecies spindle scaling

As in the case of the nucleus, studies comparing *X. laevis* and *X. tropicalis* egg extracts have illuminated mechanisms of spindle size control. *X. tropicalis* spindles are shorter than *X. laevis* spindles (Brown *et al.*, 2007), correlating with higher MT severing activity in *X. tropicalis* egg extract (Fig. 3A). Specifically, *X. tropicalis* katanin, a MT severing enzyme, was shown to be more active than *X. laevis* katanin due to the absence of an inhibitory phosphorylation site in the *X. tropicalis* katanin p60 catalytic subunit (Fig. 1). Katanin p60 depletion from *X. tropicalis* extract resulted in longer spindles that could be rescued by addition of wild-type *X. tropicalis* p60 or *X. laevis* p60 with its inhibitory phosphorylation site mutated to alanine. These results demonstrate how species-dependent phosphorylation of a single protein can affect spindle size (Loughlin *et al.*, 2011). TPX2 also contributes to the interspecies difference in spindle size, through its interaction with the kinesin-5 motor protein Eg5. Threefold higher TPX2 levels are found in *X. tropicalis* egg extract, and addition of recombinant TPX2 to *X. laevis* egg extract was sufficient to reduce spindle length (Helmke and Heald, 2014).

While cytoplasmic composition is an important determinant of spindle length, DNA amount only slightly affects spindle length. Egg extract spindles assembled with *X. tropicalis* sperm were ~10% shorter than those assembled with *X. laevis* sperm, likely due to *X. tropicalis* having approximately half the genome size of *X. laevis* (Brown *et al.*, 2007). These findings are consistent with the observations that spindles in diploid versus haploid embryos are on average only 10% longer (Wuhr *et al.*, 2008), and the same modest difference in length was also found in comparing spindles before and after pairwise fusions in *X. laevis* extracts (Gatlin *et al.*, 2009). A small increase in spindle length was also seen when the amount of chromatin was modulated on the surfaces of patterned, chromatin-coated beads (Dinarina *et al.*, 2009). In these experiments, a ~5x increase in chromatin mass on the bead surface resulted in only a ~10% increase in spindle length. Interestingly, changing the spatial arrangement of chromatin seems to have more marked effects on spindle shape than on spindle size (Dinarina *et al.*, 2009, Gaetz *et al.*, 2006).

Mechanisms of developmental spindle scaling

Developmental spindle scaling is another unexplained biological phenomenon. In early stages of *X. laevis* embryonic development, spindle length is ~60 μm and is maintained through several divisions at this upper limit irrespective of cell size (Wuhr *et al.*, 2008). By the seventh cleavage, a scaling relationship between cell and spindle size begins to emerge, in which smaller cells exhibit shorter spindles. This scaling relationship is evident when comparing differing size cells both between and within developmental stages (Wuhr *et al.*, 2008). Though it has become clear that changes in cytoplasmic volume are sufficient to drive spindle length scaling (Good *et al.*, 2013, Hazel *et al.*, 2013), there is evidence that changes in developmental cues are also involved. In studies utilizing *X. laevis* embryo extracts, the reduction in spindle size between stage 3 and stage 8 was shown to correlate with an increase in the activity of the MT-destabilizing kinesin-13 kif2a (Wilbur and Heald, 2013). While the total level of kif2a was similar in stage 3 and stage 8 embryos, in stage 3 extracts kif2a was bound to importin α and inhibited. At stage 8, sequestration of importin α into a membrane pool resulted in de-inhibition of kif2a, leading to increased MT catastrophe frequency, less stable MTs, and smaller spindles (Wilbur and Heald, 2013). These data support the idea that changes in developmental context can contribute to altered spindle size (Fig. 1, Fig. 3B).

While studies in bulk embryo extracts have revealed developmental regulators of spindle size, these types of experiments fail to capture information about the potential contribution of cell size and cytoplasmic volume to spindle size. Interestingly, spindle size can vary within the same developmental stage where cell sizes can differ dramatically. For example, in stage 8 embryos spindles are ~20 μm long in small animal pole cells, while ~50 μm long spindles are found in large cells of the vegetal pole (Wilbur and Heald, 2013). To test how cell size might affect spindle size, studies were conducted combining *Xenopus* cell-free extracts with microfluidic technology, discussed in greater detail later in this review. Using microfluidic devices to confine spindle assembly to egg extract droplets of defined sizes, it was demonstrated that spindle size correlated with droplet size (Fig. 3C), thus supporting the idea that spindle size is determined in part by cytoplasmic volume (Good *et al.*, 2013, Hazel *et al.*, 2013).

What spindle assembly component(s) might become limiting for spindle size in small cytoplasmic volumes? Tubulin itself is one possibility, given that it is the major building block of the spindle (Good *et al.*, 2013). Other potential limiting components include the flux-generating kinesin Eg5 (Burbank *et al.*, 2007, Miyamoto *et al.*, 2004), which maintains spindle length in part by producing an outward force on spindle poles (Dumont and Mitchison, 2009), and the MT polymerase XMAP215, which was recently found to regulate spindle length in a concentration-dependent manner in *X. laevis* egg extracts (Reber *et al.*, 2013). XMAP215 is particularly intriguing because, at least in *X. laevis*, it is present at relatively low concentrations (~120 nM) even before partitioning into the spindle, and small changes in protein concentration affect spindle size (Reber *et al.*, 2013). XMAP215 promotes MT growth in *X. laevis* egg extract (Gard and Kirschner, 1987, Tournebize *et al.*, 2000) and is expressed in *Xenopus* embryos from oogenesis through tadpole stages with proposed roles in regulating MT dynamics during rapid cell divisions as well as in development of the nervous system (Becker and Gard, 2000). By varying the XMAP215 concentration in *X. laevis* egg extracts, it was demonstrated that spindle size scaled with XMAP215 levels (Fig. 3D), and mutating XMAP215 to alter its polymerase activity also resulted in concomitant changes in spindle length (Reber *et al.*, 2013). XMAP215 activity is thought to regulate spindle size by promoting increased MT growth velocity, thereby affecting total MT mass within the spindle. This mechanism of action is consistent with XMAP215 being a potential effector of spindle scaling, but whether it becomes limiting during spindle assembly in small cytoplasmic volumes remains unknown. Ultimately, in order to determine which candidate proteins might be bona fide scaling factors, it will be important to measure how modulating their levels affects the cell-spindle size scaling curve. It will also be important to measure how each partitions between the cytoplasm and spindle in limiting cytoplasmic volumes.

Given that spindle size scales during development, a related question is whether there is scaling of mitotic chromosome size. Interestingly, no interspecies scaling of mitotic chromosomes was observed comparing *X. laevis* and *X. tropicalis* egg extracts, in which assembled chromosomes exhibited similar average lengths and widths. On the other hand, during *X. laevis* development mitotic chromosome length was observed to decrease between stages 7 to 11 while width remained unchanged. From stage 11 to 20, both chromosome length and width decreased (Kieserman and Heald, 2011). Concomitant scaling of mitotic chromosome and spindle lengths may help to ensure complete chromosome segregation in small cells. Interestingly, mitotic chromosome scaling does not appear to correlate with nuclear scaling during early stages of *Xenopus* development. However, it was noted that blocking expansion of nuclei assembled in egg extract resulted in smaller mitotic chromosomes, suggesting nuclear volume can influence chromosome size (Hara *et al*., 2013), consistent with chromosomal size scaling observed in *C. elegans* (Ladouceur *et al*., 2015) and defective chromosome segregation in fission yeast with small nuclei (Takemoto *et al*., 2016).

Regulation of spindle shape

Another fundamental question is how the dynamic assembly of spindle MTs is able to achieve a bipolar spindle-like shape. While centrosomes are clearly important, during female meiosis in vertebrates, bipolar spindles assemble around chromatin in a centrosome-independent manner. In meiotic egg extracts, this process is often spatially localized around exogenously added sperm nuclei or chromatin-coated microbeads (Heald *et al*., 1996). MTs nucleated around chromatin beads are able to self-organize into a bipolar spindle-like structure through the combined action of multiple different MT motor proteins (Walczak *et al*., 1998). A robust spindle pole focusing mechanism was demonstrated in experiments where forced fusion of two pre-assembled spindles resulted in a single bipolar structure (Gatlin *et al*., 2009). This de novo pole focusing also occurs in mitotic somatic cells that have been stripped of centrosomes using targeted laser ablation, indicating centrosomes are not required to ensure spindle bipolarity (Khodjakov *et al*., 2000).

Proper bipolar spindle shape is essential for cell division in normal and cancerous cells. Many cancers contain cells with supernumerary centrosomes (Chan, 2011), which pose a unique challenge to the cell division machinery. In order for these cells to divide and produce viable progeny, centrosomes must be clustered during prometaphase to avoid a multipolar anaphase, massive aneuploidy, and daughter cell death (Ganem *et al*., 2009). Multipolar divisions are very rare because cancer cells will cluster multiple centrosomes into only two poles, overcoming multipolar chromosome segregation at anaphase (Godinho *et al*., 2009). Cell-free *Xenopus* egg extract, particularly in combination with microfluidic-based encapsulation strategies, represents a promising system to study the mechanism of centrosome clustering and the transition from multipolar to bipolar spindles, as the number of microtubule organizing centers (MTOCs), either in the form of isolated centrosomes or artificial MTOCs (Tsai and Zheng, 2005), in a given volume can be easily modulated.

Relationship between size scaling of the spindle and nucleus

Do common mechanisms regulate size scaling of the spindle and nucleus? Generally, the sizes of both the nucleus and spindle scale with cell size, in other words smaller cells tend to have both a smaller nucleus and spindle. For example, smaller spindles (~10 μm pole to pole distance) and nuclei (~10 μm in diameter) are present in HeLa cells compared to spindles (~40 μm) and nuclei (~20 μm) assembled in *X. laevis* egg extract. One activity that is important for both spindle and nuclear function is the small GTPase Ran. While Ran is a key regulator of nucleocytoplasmic transport in interphase, it also facilitates spindle assembly in mitosis when RanGTP binds importin family nuclear transporters and releases spindle assembly factors (SAFs) essential for MT nucleation and assembly of a bipolar spindle. Chromatin-bound guanine nucleotide exchange factor RCC1 converts RanGDP to RanGTP generating an enrichment of RanGTP and activated SAFs around the chromatin (Fig. 1) (Caudron *et al*., 2005, Kalab *et al*., 2002). Since RanGTP and associated SAF gradients correlate with spindle and nuclear size (Kalab *et al*., 2006), one possibility is that nuclear size predefines the gradient by setting the range of the matrix over which the spindle assembles or by pre-accumulating SAFs according to nuclear size during interphase. Interestingly, in mitosis slow diffusion of former nucleoplasmic components, mediated by a membranous "spindle envelope," results in their prolonged residence within the vicinity of the forming spindle, providing another mechanism whereby nuclear size might impact spindle size (Pawar *et al*., 2014, Schweizer *et al*., 2015).

Another parallel between spindle and nuclear scaling involves importin α, whose levels impact the sizes of both nuclei and spindles. However, the mechanisms of action differ; importin α affects nuclear size by regulating bulk nuclear import (Levy and Heald, 2010), while in the case of the spindle it binds and inhibits the MT-destabilizing activity of Kif2a (Fig. 1) (Wilbur and Heald, 2013). As already discussed, the importin α cargo lamin B3 (LB3) affects interphase nuclear size. In mitosis, LB3 was observed to form a matrix-like network that scaffolds SAFs in a RanGTP-dependent but not MT-dependent manner. Interestingly, spindle assembly was disrupted in *Xenopus* egg extract depleted of LB3 or upon addition of a LB3 assembly mutant, giving rise to spindles with unfocused poles, asters, or half spindles, due to failure to assemble a spindle matrix and recruit key SAFs such as Eg5 and NuMa (Tsai *et al*., 2006). Open questions are whether LB3 levels might scale spindle size by defining the size of the matrix-like network (Shi *et al*., 2014) and whether chromatin organization might contribute to the shaping of spindle molecular gradients.

MT dynamics have been implicated in size scaling of both the spindle and nucleus. Total MT mass and growth velocity driven by XMAP215 levels were reported to define spindle size (Reber *et al*., 2013), and modulation of MT severing and depolymerization can also impact spindle size (Loughlin *et al*., 2011, Wilbur and Heald, 2013). Such effects on MT dynamics could also influence rates of nuclear expansion driven by dynein-dependent transport of nuclear-targeted membrane, discussed later in this review (Hara and Merten, 2015). As already cited, MT dynamics at the end of mitosis are also relevant to nuclear morphology, as depletion of Dppa2 from *Xenopus* egg extract gives rise to small, misshapen nuclei (Xue *et al*., 2013). Therefore, proper nuclear morphology depends on spatio-temporal control of MT network dynamics. Future studies will address whether nuclear size might program spindle size by defining physical assembly dimensions, chromatin organization, and/or SAF concentration gradients.

Xenopus cell-free extracts to study size regulation of other subcellular structures

Xenopus egg extracts support assembly of ER networks, and thus provide a useful platform to study ER morphology, dynamics, and size (Dreier and Rapoport, 2000, Voeltz *et al.*, 2006). Cell cycle regulated changes in ER structure have been studied in *Xenopus* egg extracts, where ER tubules and sheets dominate in interphase and mitosis, respectively. In interphase, ER formation starts with the concentration of membrane at MTOCs by dynein-dependent transport, followed by the outward spreading of ER tubules through association with the plus ends of growing MTs. Although MTs are not required for the formation of an ER network, when present they dictate the spatial organization of the ER. In mitosis, connections between the ER and MTs are reduced (Wang *et al.*, 2013).

As already discussed, the *Xenopus* system has been used to demonstrate how ER structure impacts nuclear size (Anderson and Hetzer, 2007, Anderson and Hetzer, 2008, Jevtic and Levy, 2015). ER networks formed in egg extract might be used to study ER functions such as protein folding, vesicular transport to the Golgi, and lipid synthesis, all activities with potential links to size scaling of other membrane-bound organelles that are interconnected with the ER. It has also been reported that Golgi and mitochondria can be reassembled in *Xenopus* egg extract (Lu *et al.*, 2006). It is important to understand the mechanisms that regulate the sizes of the ER and other interconnected organelles, as their sizes and activities often change in response to environmental signals and stress. For example, limited protein folding capacity of the ER plays an important role in the pathogenesis of many diseases, including diabetes mellitus, viral infections, retinitis pigmentosa, and Alzheimer's disease (Lin *et al.*, 2008).

Microfluidic technology and *Xenopus* cell-free extracts

Microfluidic technology has great potential to advance our understanding of size scaling of subcellular structures. With microfluidic-based encapsulation, cytoplasmic extract droplets can be generated in microfluidic devices, allowing for exquisite control of droplet size and geometry. In combination with microscopy, this approach enables one to assess the effects of boundary confinement and volume on the size and shape of *in vitro* assembled structures. The basic device we employ is a T-junction droplet generator with two inlets. An oil-surfactant mixture pumped through one inlet merges with *Xenopus* egg extract injected through a second inlet, forming extract droplets. The dimensions of the channels at the T-junction and relative flow rates of the two phases determine droplet volume, while dimensions of the droplet collection reservoir dictate droplet shape. Such experiments demonstrated that spindle size is sensitive to cytoplasmic volume and not shape (Fig. 3C), recapitulating scaling of spindle size observed during early *Xenopus* embryogenesis (Good *et al.*, 2013, Hazel *et al.*, 2013). Future applications of these droplet-generating devices include studies of nuclear scaling and ER organization by cytoplasmic volume (Fig. 2E).

Nuclear scaling has been investigated using a related technique involving confinement of *Xenopus* egg extract and preassembled nuclei in microfluidic channels of defined dimensions. By this approach, nuclear growth was observed to positively correlate with channel dimensions, suggesting that cytoplasmic volume is an important determinant of nuclear size. Above a certain threshold volume of accessible cytoplasm, nuclear size reached a plateau, corresponding to the available space occupied by the MT centrosomal aster localized to, and emanating away from, the nucleus. The volume occupied by this aster was proposed to limit dynein-mediated transport of membrane to the nucleus, thereby limiting nuclear expansion (Hara and Merten, 2015). As continuous microfluidic channels were used in these experiments, how precise modulation of cytoplasmic volume regulates nuclear growth is an open question. It will also be important to determine the relative contributions of the ER network and dynein-MT asters to nuclear size control in this system.

Xenopus egg extract and microfluidic technology have also been employed to study cytoskeletal organization outside the specific context of spindle and nuclear assembly. Confinement of *Xenopus* extract in spherical geometries with an oil boundary led to the polymerization of actin ring-like structures (Pinot *et al.*, 2012). The actin organization varied with droplet size, exhibiting a linear correlation between droplet diameter and size of the actin ring, indicating actin self-organization scales with droplet size. This resembled symmetry breaking similar to what happens when intracellular actin networks under actomyosin tension are released and only actin flow can be observed (Paluch *et al.*, 2006). Similarly, it was shown that droplet size and shape can have a profound effect on the spatial organization of MT assemblies (Pinot *et al.*, 2009).

Microfluidic technology can be further employed to study spindle and nuclear positioning within the cell. Sea urchin embryos encapsulated within microfluidic chambers of varying shapes always position their nuclei at the center of mass utilizing the MT, rather than actin, cytoskeleton. Dynein-dependent pulling forces on MTs are proposed to mediate this nuclear positioning, with pulling forces being directly proportional to MT length, thus explaining the centering of the nucleus along the longest MTs in the cell (Minc *et al.*, 2011). The scaling of such mechanical forces as a function of confinement might be studied with extract droplets that have a functionalized cortex, mimicking the cellular cortex and allowing for easy manipulation of cytoplasmic volume, composition, and geometry. A minimal system might include chemical modification of the oil/surfactant droplet periphery to resemble a lipid bilayer capable of recruiting dynein (Roth *et al.*, 2014). In summary, microfluidic technology coupled with cell-free extracts has the potential to greatly expand our understanding of not only organelle size scaling, but also how cell size and shape affect self-organization of the cytoskeleton.

Concluding remarks

The *Xenopus in vitro* cell-free system has been an invaluable platform in shaping our current understanding of how nuclear and spindle size are regulated. In this review, we discussed how species- and developmental-specific cues as well as cell size, cytoplasmic volume, and DNA amount influence size scaling of both the spindle and nucleus. Common factors that regulate size and function of both the spindle and nucleus include the small GTPase Ran, importin α, lamins, TPX2, and MT dynamics. It remains to be elucidated if a causative relationship between nuclear and spindle size exists and whether nuclear size might program spindle size. Emerging technologies, such as microfluidics (Liu and Singh, 2013), advances in microscopy (Zumbusch *et al.*, 2013), and high-throughput imaging (Shachar *et al.*, 2015) greatly

expand the possibilities for studying organelle size scaling in the *Xenopus* extract system.

Our improved mechanistic understanding of organelle size regulation will facilitate future studies into the functional significance of nuclear and spindle size during normal development and differentiation. Furthermore, it is now becoming possible to investigate how altered organelle morphology contributes to diverse disease states such as cancer, progeria and other laminopathies, and disorders associated with misfolded proteins (Godinho *et al.,* 2009, Lin *et al.,* 2008, Yazdani *et al.,* 2012, Zwerger *et al.,* 2011). Notably, cancer cells with increased nuclear size generally exhibit perturbed higher order chromatin structure that can contribute to increased mutation frequencies (Schuster-Bockler and Lehner, 2012). There are important open questions about the potential link between altered nuclear size and chromatin organization, the contribution of oncogenes and tumor suppressors to nuclear enlargement, and whether reducing nuclear size in cancer cells might reverse tumorigenic potential. The *Xenopus* cell-free extract system will continue to be an important tool in answering fundamental questions about the regulation of organelle morphology, answers that promise to provide novel approaches to disease diagnosis, prevention, and treatment.

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