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REVIEW

Understanding eukaryotic chromosome segregation from a comparative biology perspective

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ABSTRACT

A long-appreciated variation in fundamental cell biological processes between different species is becoming increasingly tractable due to recent breakthroughs in whole-genome analyses and genome editing techniques. However, the bulk of our mechanistic understanding in cell biology continues to come from just a few well-established models. In this Review, I use the highly diverse strategies of chromosome segregation in eukaryotes as an instrument for a more general discussion on phenotypic variation, possible rules underlying its emergence and its utility in understanding conserved functional relationships underlying this process. Such a comparative approach, supported by modern molecular biology tools, might provide a wider, holistic view of biology that is difficult to achieve when concentrating on a single experimental system.

KEY WORDS: Chromosome segregation, Comparative biology, Evolution, Mitosis, Nuclear envelope, Spindle

Introduction

The generality of cell biological mechanisms has become a go-to argument for researchers explaining the relevance of their work to funding bodies, scientific journals and the general public. We often argue that we study cell division in yeast, cell polarity in worms and cell migration in flies because these are simple experimentally tractable models. The corollary is that once we understand the process of interest in a model, we may start making strong inferences about more-complex and less-tractable systems, which are often understood to be humans or organisms important for humans. Indeed, model organisms, the term that by now has come to mean those 'for which a wealth of tools and resources exist' (Russell et al., 2017), have been invaluable in delineating the components and the logic of cell biological programs.

However, every model organism has a unique life history and is ultimately a model for itself. Focusing on mechanistic commonalities conceals this biological diversity and the processes underlying its emergence. Different eukaryotes tend to have overlapping but distinct sets of genes, and this fact alone might account for variability even in the most fundamental biological pathways. Changes in protein coding potential, gene regulatory capacity and non-coding regions of the genome lead to further divergence. As a result, virtually every cell biological process likely exists in a continuum of states with multiple ways to achieve a specific functional goal. Understanding variation at a cellular level may provide insights into how functional innovations arise during evolution and illuminate hidden links between the process of interest and the rest of cellular physiology and ecology. Conversely, it may additionally uncover the core 'conserved' components and relationships in cell biological machineries.

Eukaryotic cells compartmentalize the genome within the nucleus delimited by the nuclear envelope (NE). During both mitosis and meiosis, chromosomes are segregated by a bipolar microtubule-based spindle. Faithful chromosome partitioning depends on the following key elements: (1) the centromeres, which are the *cis*-elements on the chromosomes responsible for their transmission (see Glossary); (2) the kinetochores (see Glossary), a trans-acting apparatus connecting sister centromeres with microtubules originating from the opposing poles of the spindle; (3) a way to restructure the mother NE and allow the spindle to access the chromosomes; and (4) assembly of a functional spindle. Surprisingly, for something so intrinsic to biological fitness and survival, chromosome segregation strategies and corresponding molecular machineries have diverged considerably in evolution. In this Review, I will discuss these key steps of chromosome segregation in the light of biological diversity. This framework will also allow me to cover some relevant approaches, from comparative studies of related species to addressing cell biological questions using methods of phylogenomics and population genetics.

Comparative biology of centromere function

Initially defined as sites of spindle microtubule attachment, centromeres often appear under the microscope as narrowed regions, the primary constrictions of mitotic chromosomes. The primary constrictions may differ in length, and, indeed, some so-called holocentric species lack them altogether, attaching microtubules along the entire length of the chromosome (Fig. 1A). How do centromeres arise and why does centromere architecture show such a remarkable degree of divergence, such that it is evident even in closely related species?

How do DNA sequence-defined 'point' centromeres arise?

The first centromere characterized at the molecular level originated from the budding yeast *Saccharomyces cerevisiae* (Clarke and Carbon, 1980; see Meraldi et al., 2006 and Biggins, 2013 for reviews). A short ∼120-bp so-called CEN sequence containing an AT-rich CDEII region flanked by the binding sites for CBF1 and CBF3 protein complexes is necessary and sufficient for DNA inheritance in this organism (Fig. 1B). Interestingly, the basic helix-loop-helix protein CBF1, which interacts with the CDEI element, also functions as the transcription factor that recognizes similar landing sites throughout the genome, suggesting that its promoter and/or enhancer-binding activity might have been repurposed for centromere definition (Cai and Davis, 1990). Kinetochore assembly at the CEN sequence requires binding of the budding yeast-specific CBF3 protein complex to the CDEIII element (Lechner and Carbon, 1991), followed by the recruitment of an unusual histone H3 variant from the CENP-A family to the

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Glossary

Centromere: a *cis*-element on the chromosome responsible for its transmission. Point centromeres are based on specific DNA sequences, whereas regional centromeres contain long stretches of AT-rich, repetitive DNA and are defined epigenetically. A neocentromere is defined as a chromosomal locus away from the centromere that acquires centromere identity *de novo*, usually following chromosome breakage or inactivation of the original centromere.

Centromere drive: a hypothesis postulating that an asymmetry in female meiosis, where only one out of the four meiotic products is included into the egg nucleus, may cause a genetic conflict between the centromeric DNA and proteins that bind to it to promote its segregation (Henikoff et al., 2001). In this model, centromeric DNA acts selfishly to be partitioned to the egg (e.g. by expanding the number of repeats) and therefore, increasing centromere 'strength'. Following an episode of the drive, an imbalance in the strength of centromeres may become deleterious in the context of symmetric male meiosis. It is hypothesized that centromeric proteins may evolve adaptively to suppress such an imbalance and restore the parity.

Kinetochore: a large protein complex that connects centromeric DNA with spindle microtubules. In addition to their direct role in chromosome partitioning, kinetochores also serve as signaling centers that monitor the accuracy of chromosome attachment to spindle microtubules, restricting cells from progressing into anaphase until all errors are corrected.

LECA: the last eukaryotic common ancestor, an organism postulated to be the first eukaryote, which has been reconstructed through molecular phylogeny methods (for details see Koonin, 2010). Predicted to possess a sophisticated eukaryotic cell biology toolbox.

Subcellular scaling: positive correlation of the size, the number and/or functional capacity of organelles and subcellular structures with cell size.

CDEII sequence (Meluh et al., 1998; Stoler et al., 1995). The CEN sequence is occupied by one CENP-A-containing nucleosome and binds to one spindle microtubule (Furuyama and Biggins, 2007; Krassovsky et al., 2012). Such a 'point' centromere, which is defined by the DNA sequence and recognized by specific protein complexes (Fig. 1B, left), was later shown to be an exception rather than the rule and has been thought to have originated only once, in a relatively recent ancestor of the budding yeast.

However, it appears that the DNA sequence-based centromere is in fact highly evolvable (Kobayashi et al., 2015). *Naumovozyma castellii*, a distantly related budding yeast, exhibits a strikingly different AT-rich point centromere that is organized in a three-element pattern. Even though the CDEIII sequences are clearly different between *N. castellii* and *S. cerevisiae*, they both recruit CBF3 (Fig. 1B, right). Consistent with a possible adaptation to the new centromeric sequence, the DNA-binding domain of Ndc10, one of the CBF3 subunits recognizing consensus elements, has evolved to a greater extent as compared with its homologs in budding yeasts with the 'conventional' CEN. It appears that the unusual centromeric sequence has not simply replaced the original CEN but arose *de novo*, and eventually propagated to all chromosomes, superseding the conventional centromeres (Kobayashi et al., 2015). We do not yet understand how the new centromeric sequences arise and what could be the reasons for their successful transmission. Analyzing the centromere dynamics in inter-species hybrids (Marinoni et al., 1999) or reconstituting an *N. castelli* centromere in *S. cerevisiae* and observing how the system evolves in the laboratory, could provide an experimental handle on the problem. It remains to be seen whether point centromeres exist in other eukaryotic lineages and, if so, what could be the rules underlying the emergence of these functional elements.

Curiously, although completely different on the cytological level, the holocentric chromosomes in the round worm *Caenorhabditis elegans* may contain many dispersed point centromeres (Fig. 1C). These CENP-A-bound sites coincide with 15-nt GA-rich high-occupancy target (HOT) motifs that serve as low-affinity landing sites for many transcription factors (Steiner and Henikoff, 2014). Low-affinity binding of HOT spots by transcription factors and/or exclusion of conventional nucleosomes may help to maintain the functional identity of these regions. Thus, at least in this organism, holocentricity may have arisen through co-opting a fairly generic transcription factor-binding motif, with properties that favor binding of CENP-A, into a polycentromeric array (Steiner and Henikoff, 2014). It is currently unknown whether centromeres in other holocentric organisms are organized in a similar manner. Related species with either monocentric or holocentric chromosomes, such as Cuscuta plants (Zedek and Bureš, 2017), could be used to probe a number of exciting questions that have remained largely out of reach of experimental research. For example, how does holocentricity arise? Could it be adaptive under certain circumstances? What are the evolutionary constraints associated with holocentricity?

How do 'regional' centromeres evolve?

Most organisms do not appear to rely on specific DNA sequences to define their centromeres but instead organize them epigenetically around large stretches of AT-rich, often repetitive, DNA; for example, tandem arrays of α-satellites in primates (Vafa and Sullivan, 1997). Some of the satellites contain short binding sites for CENP-B, a domesticated pogo-like transposase (Aldrup-Macdonald and Sullivan, 2014) (Fig. 1D). The repetitive nature of such 'regional' centromeres lends itself to extremely rapid evolution, with frequent repeat expansion, contraction and homogenization events (Kalitsis and Choo, 2012; Montefalcone et al., 1999). Evolutionarily new centromeres (Giulotto et al., 2017; Piras et al., 2010; Wade et al., 2009) or abnormal neocentromeres (Marshall et al., 2008) can be formed on non-repetitive DNA, suggesting that tandem repeats are not strictly essential for centromere determination. Rather, they may facilitate centromeric function, as neocentromeres often exhibit errors in kinetochore–microtubule attachment (Bassett et al., 2010) and evolutionarily new centromeres tend to accumulate α-satellites (Rocchi et al., 2012).

The common denominator for all the diverse centromeric structures is usually thought to be the assembly of CENP-A nucleosomes. CENP-A is found throughout the eukaryotic tree of life; it is essential for chromosome segregation and sufficient for organization of functional kinetochores (Barnhart et al., 2011; Guse et al., 2011; Mendiburo et al., 2011). Within the regional centromeres, only a small proportion of nucleosomes carry CENP-A (Fig. 1D), but given that CENP-A-containing nucleosomes are overrepresented at the centromeres as compared with at the rest of the genome, this appears sufficient to define an active centromere epigenetically (Bodor et al., 2014). CENP-A often exhibits signatures of adaptive evolution even in closely related species (Malik and Henikoff, 2001), possibly because it interacts with the rapidly evolving centromeric DNA.

Thus, in spite of the paramount requirement for accurate chromosome segregation, both centromeric DNA and its associated CENP-A histone variant show rapid evolutionary divergence. The 'centromere drive' hypothesis (see Glossary) explains this phenomenon by postulating that both DNA and protein components of the centromere evolve under the conditions of a genetic conflict (Henikoff et al., 2001). In the asymmetric female meiosis, where only one meiotic product out of four is included into the oocyte, centromeric DNA sequences could compete for preferential partitioning into the gamete. Mutations conferring any advantage, for instance the expansion of the repeat array providing

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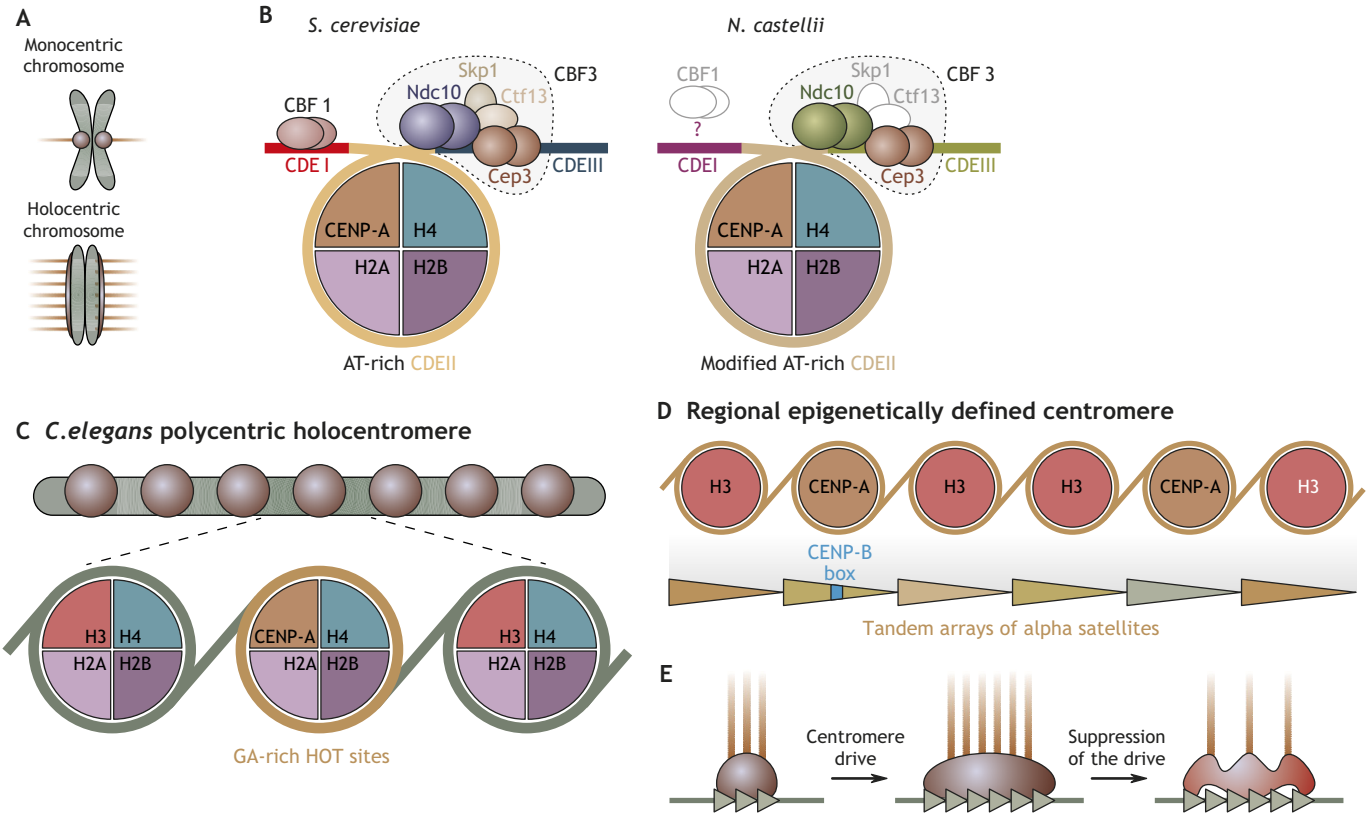

Fig. 1. Centromere architecture in eukaryotes. (A) Overall configuration of monocentric and holocentric chromosomes during mitosis. Spindle microtubules emanating from the kinetochores assembled at centromeric sites are also shown. (B) Left: centromere structure in *S. cerevisiae*. A ∼120-bp sequence contains an AT-rich CDEII region (in beige) that is flanked by two conserved sequence elements, CDEI and CDEIII. The CDEII DNA wraps around a CENP-A-containing nucleosome, with the histones H2A, H2B and H4 also indicated. The CBF1 homodimer binds to CDEI, and the CBF3 complex, consisting of an Ndc10 homodimer, a Cep3 homodimer, and Skp1 and Ctf13, binds to CDEIII. Right: a 110-bp centromere in *N. castellii*. Here, a diverged AT-rich CDEII region is flanked by CDEI and CDEIII elements, which are unrelated to their *S. cerevisiae* counterparts. Ndc10, but not the other CBF3 DNA-binding component Cep3, exhibits a signature of adaptive evolution. The roles of Skp1 and Ctf13 in CBF3 assembly have not been addressed to date. It also remains to be seen whether CDEI is regulated by CBF1 binding in this organism. (C) Schematic illustration of the proposed polycentric, rather than the diffusive, holocentromere structure in *C. elegans*. Note that the centromeric activity centers on one CENP-A nucleosome that is positioned at GA-rich high-occupancy target (HOT) motifs; this is flanked by two histone H3-containing nucleosomes. (D) Illustration of a regional epigenetically defined centromere, modeled on human centromeres. The underlying DNA (shown below) consists of long head-to-tail arrays of so-called α-satellites, ∼170-bp-long AT-rich repeats of varying sequence conservation (indicated by the slightly different colors). Some of the satellites contain binding sites for CENP-B. Relatively rare CENP-A-containing nucleosomes (golden) are interspersed between the conventional ones (dark red). (E) A diagram outlining the genetic conflict between the centromeric DNA and centromeric proteins, which underlies the centromere drive hypothesis. Centromere 'strength' (i.e. binding to spindle microtubules; depicted in ochre), may increase due to the satellite (gray-green triangles) array expansion causing increased recruitment of centromeric proteins (shown in chocolate) and assembly of larger kinetochores. The drive can be suppressed if the centromeric proteins evolve to have a weaker binding to DNA (mutant version depicted on the right).

more CENP-A-organized microtubule interfaces, would allow this particular chromosome to be inherited. However, in the symmetric male meiosis, such an imbalance in centromere strengths could lead to defects in chromosome segregation. Mutations in CENP-A that restore the balance, by modulating its interaction with DNA, would be selected (Fig. 1E). The hypothesis postulates that successive episodes of centromere drive would result in rapid co-evolution of centromere components. Importantly, it predicts that crosses between isolated populations of the same species with independently diverged centromeres would produce defective hybrids, thus contributing to speciation (Henikoff et al., 2001).

The comparative approach has been invaluable in testing and refining this model. For instance, in the closely related species of *Solenopsis* fire ants, the centromeres are made up of CenSol satellite arrays (Huang et al., 2016). However, the number of repeats differs dramatically between the species, with CenSol arrays expanded to cover approximately one-third of each chromosome in *S. invicta*. Reconstructions of the ancestral state suggest that the original centromere in this clade was short. As ant males are haploid, there should be no conflict between centromeres of paired chromosomes in male meiosis. Thus, in haplo-diploid organisms, such as some insects, the centromeric satellite repeats may undergo runaway expansion due to uncontrolled centromere drive (Huang et al., 2016). However, analysis of *Mimulus* monkey flower, a genus with the well-documented female meiotic drive, identified events of CENP-A duplication and paralog subfunctionalization in closely related species, consistent with an antagonistic evolutionary relationship between centromeric DNA and its interacting proteins (Finseth et al., 2015). Similarly, several independent CENP-A duplications have occurred within the *Drosophila* genus, with clear instances of positive selection acting on some paralogs. Following gene duplication, one paralog may evolve as a drive suppressor in the male germline, whereas the other could continue executing conserved mitotic function in somatic cells, effectively separating protein functions with divergent fitness optima (Kursel and Malik, 2017).

Reconstituting the divergent process in sister species may be an ultimate test for many hypotheses that have been posed based on rich phylogenomics and genetics data. The CENP-A proteins from different *Drosophila* species exhibit a signature of positive selection in the loop 1 (L1) region thought to interact with DNA, and the CENP-A from *D. bipectinata* does not localize to *D. melanogaster* centromeres (Vermaak et al., 2002). These two observations suggesting that CENP-A evolved adaptively to bind to ever changing centromeric DNA, were used in support of the original centromere drive hypothesis (Henikoff et al., 2001). However, it turned out that CENP-A L1 co-evolved adaptively with the N-terminus of the CENP-A chaperone CAL1, and it was this interaction that modulated the efficiency of CENP-A loading onto centromeric DNA (Rosin and Mellone, 2016). These results do not explicitly exclude the possibility that there could have been CENP-A-based drive episodes during *Drosophila* evolution, but they indicate that the centromere drive can be compensated for through multiple means. Indeed, CENP-A in plants and mammals has evolved under negative selection, which is not consistent with the proposed function in alleviating the centromere drive. Rather, it is CENP-C, the CENP-A nucleosome remodeler (Falk et al., 2015), which frequently exhibits the signature of rapid adaptive evolution (Talbert et al., 2004).

In several lineages, CENP-A has been lost altogether. In insects, recurrent CENP-A losses have occurred in lineages that have acquired holocentricity (Drinnenberg et al., 2014). However, several inner kinetochore components – which require CENP-A for centromere localization in CENP-A-containing organisms – are still present in their genomes. It remains to be seen whether these have been repurposed for other functions or have acquired a CENP-A-independent mechanism for their recruitment to centromeric DNA, presumably by interacting with a newly evolved centromeric 'reader'. Understanding how kinetochores are assembled in CENP-A-deficient lineages should provide novel insights into centromere definition and function. Importantly, this might shed light on how fundamental cellular processes are restructured in evolution, possibly through the emergence of new lineage-specific essential genes (Ross et al., 2013).

Diversity of kinetochore organization and function

Centromeres assemble the kinetochores, the large multisubunit structures that coordinate chromosome segregation during mitosis and meiosis (see Musacchio and Desai, 2017 for a review). Currently, our understanding of kinetochore diversity is based mainly on phylogenomic analyses that have singled out interesting outliers for mechanistic studies rather than on side-by-side comparisons of related species exhibiting distinct kinetochore organization and function.

How is the centromere–microtubule interface organized?

Our mechanistic understanding of kinetochore architecture comes mostly from experiments performed in yeast and mammalian cells, where the inner kinetochore complex that is assembled at CENP-A-marked centromeric DNA interacts with the outer kinetochore, which connects to spindle microtubules. The inner kinetochore is composed of several subcomplexes of the constitutive centromere-associated network (CCAN) (Musacchio and Desai, 2017). In spite of importance of CCAN in yeast and mammals, most of its components have been lost in two out of five eukaryotic supergroups and even some Opisthokonta species, including the model animal *D. melanogaster* (van Hooff et al., 2017a). CENP-C is an interesting exception, as it has been largely retained in evolution (van Hooff et al., 2017a). At least in *Drosophila*, CENP-C is capable of directly recruiting the outer kinetochore components, potentially short-circuiting the requirement for an extensive CCAN (Przewloka et al., 2011). This indicates that the bridging between the centromeric chromatin and the microtubule-binding interface can be achieved through different and not necessarily complex means, and that the functions of the CCAN are likely not limited to serving as a platform for outer kinetochore assembly (Bancroft et al., 2015; Hori et al., 2013; Osman and Whitby, 2013; Suzuki et al., 2014). Indeed, in the parasitic kinetoplastid *Trypanosoma brucei*, which assembles the kinetochores on AT-rich repetitive arrays in the absence of CENP-A and most other recognizable kinetochore proteins, the lineage-specific KKT2/3 proteins appear to have taken on this role in inner kinetochore assembly (Akiyoshi and Gull, 2014; D'Archivio and Wickstead, 2017). The reasons for this virtually complete remodeling of the kinetochore are not clear, since the genomes of euglenids, the sister group of kinetoplastids, encode CENP-A together with many conventional kinetochore proteins, and do not appear to encode the KKT complex (see Akiyoshi, 2016 for a review).

While ∼70% of the extant kinetochore proteins have been inferred to be present in the last eukaryotic common ancestor (LECA; see Glossary), individual lineages have undergone frequent gene losses, duplications and rapid evolution on the sequence level (van Hooff et al., 2017a). However, the architecture of the core outer kinetochore [i.e. the KNL1–MIS12–NDC80 (KMN) network], appears to be relatively well conserved, in particular, the NDC80 subcomplex, which links the kinetochore to microtubules (van Hooff et al., 2017a). Thus, the NDC80 kinetochore–microtubule interface likely arose in the LECA and this configuration has continued to be used throughout eukaryotic evolution.

However, the microtubule-tracking activity that allows kinetochores to maintain interaction with depolymerizing microtubules during chromosome segregation has been subject to rearrangements. The Ska tracking complex (Welburn et al., 2009), inferred to be present in LECA, may have been replaced by a completely unrelated Dam1 complex (Westermann et al., 2006) in the ancestor of fungi. This innovation appears to have subsequently spread to several non-fungal lineages through horizontal gene transfer (van Hooff et al., 2017b). Understanding the functions of Ska and Dam1 in the species predicted to contain both complexes and comparing them to sister lineages that have retained just one microtubule tracker could explain how new protein functions emerge. Some lineages lack both Ska and Dam1, suggesting that they may have evolved other tracking complexes. The recent identification of a different, completely unrelated, kinetochore–microtubule tracker (KKT4) in *T. brucei* suggests that there indeed might be many solutions to this problem (Llauró et al., 2017 preprint). Alternatively, chromosomes could be segregated through mechanisms that do not rely on microtubule tracking. For instance, in *C. elegans* meiosis, chromosomes appear to be pushed apart by inter-chromosomal microtubule arrays (Laband et al., 2017), whereas dinoflagellates employ highly unusual NE-associated kinetochores that may facilitate physical separation of chromosomes via the membrane (Bhaud et al., 2000).

Genomics-led advances in understanding mitotic checkpoint and error correction mechanisms

In addition to organizing the nuts and bolts of chromosome segregation, the kinetochore monitors its microtubule attachment status by recruiting the components of the spindle assembly checkpoint (SAC) and the attachment error correction pathway (for reviews, see Cheeseman, 2014; Haase et al., 2017; Musacchio,

2015). The components and the general logic of these pathways appear to have arisen in the LECA (Eme et al., 2011; van Hooff et al., 2017a; Vleugel et al., 2012). In mammalian cells and yeast, Mad2 (MAD2L1 in mammals), Bub1R (also known as BUB1B in mammals, Mad3 in yeast), Bub3 and Cdc20 constitute the mitotic checkpoint complex (MCC), which is generated on unattached kinetochores and functions as an inhibitor of the Cdc20-containing anaphase-promoting complex/cyclosome (APC/C-Cdc20) (Izawa and Pines, 2015). When the checkpoint is satisfied, the MCC disassembles and the APC/C is free to drive chromosome segregation and mitotic exit, for example, by ubiquitylating securin and cyclin B (see Chang and Barford, 2014 for a review).

The MCC component BubR1 and the kinase Bub1, which acts as a stable scaffold for coordinating checkpoint signaling (Elowe, 2011; Rischitor et al., 2007), are distant paralogs, produced by duplication and subfunctionalization of an ancestral MadBub gene. Strikingly, duplication has occurred independently in at least 16 different lineages, with most paralogs subject to comparable evolutionary fates (Tromer et al., 2016). The ancestral MadBub had both a kinase domain and a KEN box motif essential for APC/C-Cdc20 inhibition. Following gene duplication, one paralog (BubR1) retained the KEN box, but lost the kinase activity due to truncations or inactivating mutations, whereas the other lost the ability to inhibit APC, but retained the working kinase domain (Bub1) (Vleugel et al., 2012). Many lineages still have a single MadBub gene – in fact, the ancestral versions of MadBub can execute both BubR1 and Bub1 functions in mammalian cells and budding yeast (Nguyen Ba et al., 2017; Suijkerbuijk et al., 2012). Repeated reorganization of MadBub paralogs in evolution could have been non-adaptive owing to the relative ease of degenerative mutations that remove functional features (Nguyen Ba et al., 2017). Alternatively, the separation of functions in paralogs might have been selected in evolution because of low tolerance of MadBub kinase domain for amino acid substitutions, which manifest in destabilization of mutant proteins (Suijkerbuijk et al., 2012).

Phylogenomics can predict previously unknown functional interactions by identifying protein domains and proteins that have similar species distribution, suggesting a degree of co-evolution. This approach led to the identification of a co-evolved unit containing a KEN box flanked by two ABBA motifs in BubR1, which was shown to be essential for APC/C inhibition by the SAC (Tromer et al., 2016). Another phylogenomics study pointed to a possible function of the nucleoporin Y-complex (Loïodice et al., 2004; Zuccolo et al., 2007) in SAC signaling (van Hooff et al., 2017a).

Genome-wide analyses indicate substantial variability in MCC recruitment to kinetochores and the mechanisms that subsequently silence the checkpoint (van Hooff et al., 2017a; Vleugel et al., 2012). This may reflect different levels of complexity in kinetochore–microtubule attachment (e.g. a single microtubule per kinetochore in budding yeasts versus many in vertebrates), requiring different approaches for error detection and correction. However, there could be another explanation for the rapid evolution of factors involved in MCC recruitment, such as Knl1 (Tromer et al., 2015; Vleugel et al., 2013, 2015). Perhaps counterintuitively, in female meiosis in mice, 'stronger' kinetochores that carry more centromere 'readers', and are therefore expected to attract more microtubules according to the centromere drive hypothesis, detach more frequently than those with weaker kinetochores, thereby allowing reorientation of bivalents on the spindle and preferential incorporation of stronger centromeres into an egg (Akera et al., 2017). This suggests that the spindle checkpoint and/or error correction pathways could be subject to a meiotic drive although the molecular mechanisms that would allow the DNA elements of the centromere to execute such a control remain to be elucidated.

Investigating the mechanisms underlying variant mitotic nuclear envelope remodeling

Many of the examples discussed so far have utilized a sequence-led approach where mismatches between genetic toolkits of different organisms are used as predictors of interesting new biology. An orthogonal strategy that can provide valuable insights into both variability and conservation of cell biological mechanisms relies on side-by-side comparison of closely related species. In this case, experimental tractability is the key, but virtually any research methodology is appropriate as long as it affords mechanistic understanding of the process of interest. Related species share the bulk of their genetic makeup, and it is precisely this similarity that helps in deducing the routes to biological innovation, whether it is the rewiring of networks that contain largely conserved elements or the generation of new behaviors through gene gain or loss. The beauty of this approach is that a process of interest can be not only dissected but readily reconstituted, providing additional and often unanticipated layers of biological understanding.

In my laboratory, we use this approach to understand NE remodeling during mitotic division. Over the course of evolution, eukaryotic cells have arrived at a number of solutions to restructure the NE, which allows chromosome segregation and formation of the daughter nuclei. Some cell types undergo 'closed' mitosis, leaving the NE intact and assembling an intranuclear mitotic spindle. Others break the NE for the duration of a so-called 'open' mitosis, allowing the cytoplasmic spindle to capture and partition chromosomes (see Ungricht and Kutay, 2017; Zhang and Oliferenko, 2013 for reviews). The 'modern' eukaryotes use both strategies and a number of variations in between, which differ in the timing and the extent of NE breakdown (see Makarova and Oliferenko, 2016 for a review). Suggesting that this phenotypic richness can be achieved through relatively minor tweaks to the cellular machinery regulating NE integrity and function, related organisms and even different cell types within the same organism can exhibit different approaches to NE remodeling (see Heath, 1980; Makarova and Oliferenko, 2016 for an overview).

The two fission yeasts, *Schizosaccharomyces pombe* and *Schizosaccharomyces japonicus* exemplify such a divergent pair of related genetically tractable species (Rhind et al., 2011; Russell et al., 2017). *S. pombe* is a well-established model yeast, which segregates chromosomes in a closed mitosis, whereas the much larger *S. japonicus* cell ruptures the NE during anaphase (Aoki et al., 2011; Yam et al., 2011) (Fig. 2A). As the molecular machinery supporting nucleocytoplasmic compartmentalization appears functional up to the point of NE breakage (Gu et al., 2012; Yam et al., 2013), *S. japonicus* must rely largely on the cell-cycle-driven membrane remodeling events to introduce discontinuities into the NE. This specific aspect of NE remodeling, together with post-mitotic NE reformation, make *S. japonicus* a nice simple system to probe nuclear membrane remodeling and the chromatin–NE interactions that are relevant to open mitosis. We have used *S. japonicus* to investigate the roles of the LEM-domain-containing inner nuclear membrane proteins in supporting NE integrity and association between the chromatin and the nuclear periphery (Yam et al., 2011, 2013).

However, the full promise of the above system lies in exploiting a comparative biology angle. For instance, we found that whereas the nuclear membrane surface area increases during mitosis in

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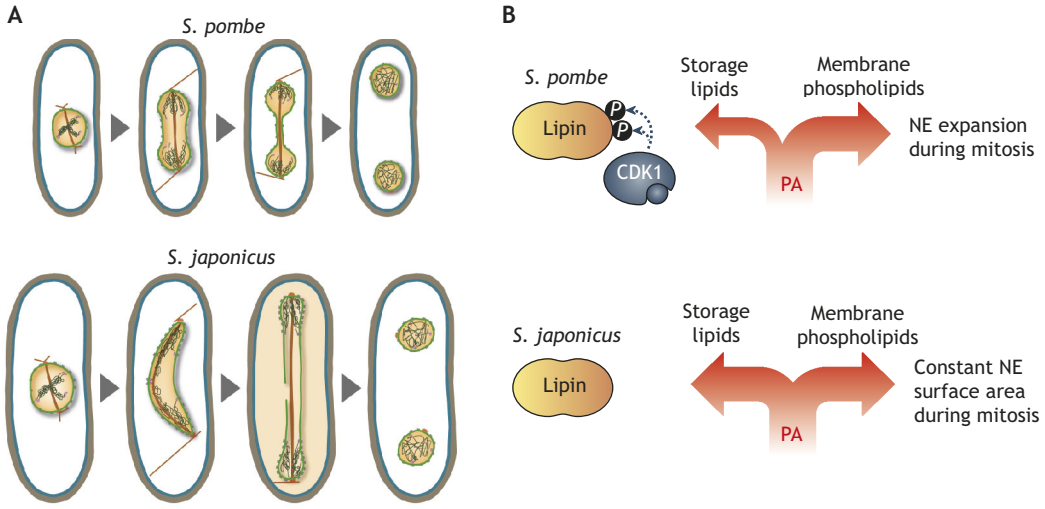

Fig. 2. Evolutionary divergence of strategies for mitotic nuclear envelope management within the fission yeast clade. (A) In *S. pombe* (top), the NE remains intact for the duration of mitosis, whereas in *S. japonicus* (bottom), it breaks during anaphase B. NE breakage is illustrated by abrupt redistribution of nucleoplasmic components (beige) throughout the cell. The mitotic spindle, chromosomes and the NE are also indicated. Note the considerably larger cell size in *S. japonicus*. Adapted from Gu and Oliferenko (2015) with permission from Elsevier. (B) Non-scalable NE expansion, which is required for 'closed' mitosis in *S. pombe*, is possible due to CDK1-driven inhibitory phosphorylation of the phosphatidic acid (PA) phosphatase lipin, which channels PA flux towards phospholipid biosynthesis. Lipin is not phosphorylated in *S. japonicus*; therefore, its NE does not expand during mitosis, which necessitates NE breakage. This figure has been adapted from Makarova et al. (2016), where it was published under a CC-BY license (https://creativecommons.org/licenses/by/4.0/).

S. pombe, it remains constant in *S. japonicus* (Yam et al., 2011). As a result, the elongating *S. japonicus* spindle buckles under a growing compressive force exerted by the NE. The spindle straightens following the NE rupture that is triggered in anaphase, allowing chromosome segregation. Tellingly, preventing mitotic NE expansion in *S. pombe* leads to comparable spindle deformations but with a strikingly different functional outcome. Since *S. pombe* does not have a mechanism for NE rupture, spindles break under compression and fail to segregate the daughter genomes. This result underscores the requirement for NE expansion to support closed mitosis but also the need for inventing a NE breakdown mechanism when such an expansion does not occur (Yam et al., 2011).

This divergence in the control of NE surface area can be explained by differential regulation of the phosphatidic acid (PA) flux by the cell cycle machinery. We discovered that, whereas the PA phosphatase lipin is inactivated by its Cdk1-dependent phosphorylation during metaphase in *S. pombe*, leading to a sharp increase in membrane phospholipid biosynthesis and NE expansion, it is not subject to such a regulation in *S. japonicus* (Fig. 2B) (Makarova et al., 2016). The evolution of lipin regulation might have occurred through the modulation of the activity of the lipin phosphatase Spo7–Nem1, which counteracts inhibitory phosphorylation events, rather than owing to the acquisition or the loss of *cis*-motifs directing Cdk1 phosphorylation (Makarova et al., 2016). Thus, the two fission yeasts with their divergent NE expansion strategies have emerged as an excellent comparative system to gain insights into the cellular control of PA flux by the evolutionarily conserved lipin–Spo7–Nem1 circuitry, which functions at the intersection of several lipid metabolic pathways in all eukaryotes (Bahmanyar et al., 2014; Csaki et al., 2013; Golden et al., 2009; Gorjanacz and Mattaj, 2009; Han et al., 2012; Kim et al., 2007; Reue and Dwyer, 2009; Santos-Rosa et al., 2005; Siniossoglou et al., 1998).

Mechanistic understanding of variability in NE management mechanisms may also help us in considering what might influence the evolution of mitosis. That said, the comparative approach in itself does not offer explicit evolutionary insights. As an example, it is certainly possible to rationalize possible reasons for keeping the NE intact or disassembling it during mitosis (Makarova and Oliferenko, 2016; Sazer et al., 2014), but the existing functional plasticity may have evolved non-adaptively or to satisfy the requirements of meiotic divisions. Of note, although *S. pombe* divides through closed mitosis, it undergoes transient loss of nucleocytoplasmic integrity during the anaphase of meiosis II, without obvious NPC disassembly or membrane breakage (Arai et al., 2010; Asakawa et al., 2010). The functional significance of such a 'virtual' NE breakdown is unclear, but it is conceivable that it could promote the restructuring of the chromatin or cytoplasmic components required for gamete formation and survival (Flor-Parra et al., 2018). In principle, if mechanisms responsible for the loss and recovery of nuclear integrity are already present in a given species owing to their function in meiosis, they could be modulated to produce a distinct mitotic program. It would be of interest to investigate whether loss of nucleocytoplasmic integrity is common for meiotic divisions, in particular in lineages that exhibit a patchwork distribution of mitotic NE remodeling strategies. Ultimately, it is the understanding of mechanisms underlying distinct NE behaviors and their connection to other parts of cellular physiology that may generate testable hypotheses for the evolution of mitosis.

Using a comparative approach to understand the scaling of the spindle apparatus

Groups of related organisms can be used as composite experimental systems to study recurring biological phenomena, such as subcellular scaling (see Glossary). As cells come in different sizes – not only between different species but also during development of an individual species – the subcellular architecture must be scaled to ensure robust operation across a vast range of cellular volumes (see Levy and Heald, 2012; Reber and Goehring, 2015 for reviews). The microtubule-based spindle apparatus is a beautiful example of a dynamic scalable assembly. During early animal development, when cells undergo successive divisions with virtually no growth in between, metaphase spindles scale with cell size, although the relationship breaks down in very large cells, with spindles reaching a maximum

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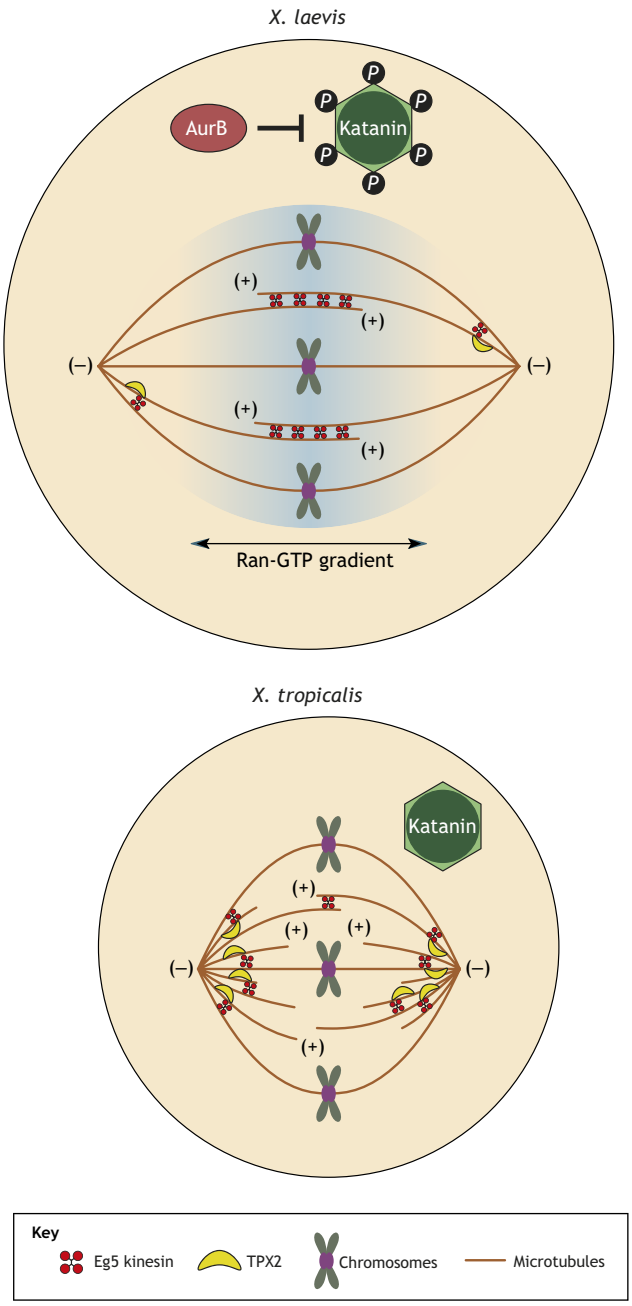

Fig. 3. Inter-species scaling of the meiotic spindle. Larger *X. laevis* eggs (top) assemble larger spindles, as compared to smaller *X. tropicalis* eggs (bottom). Note that *X. tropicalis* spindles exhibit less antiparallel microtubule overlap at the spindle equator than those in *X. laevis*. Spindle assembly in *X. laevis*, but not *X. tropicalis*, depends on the chromatin-centered gradient of Ran-GTP and the tetrameric kinesin Eg5. This difference can be attributed to higher concentration of the microtubule assembly factor TPX2 in *X. tropicalis* cells, which causes retention of Eg5 near spindle poles and reduces Eg5-mediated microtubule overlap at the spindle midzone. Additionally, the microtubule-severing enzyme katanin is inactivated by Aurora B-dependent phosphorylation in *X. laevis*, which increases microtubule stability and leads to larger spindle size. Such a phoshoregulation does not occur in *X. tropicalis*. A pictorial legend is provided to indicate main players in spindle scaling in these two species. This figure has been adapted from Helmke and Heald (2014), where it was published under a CC-BY-NC-SA license (https://creativecommons.org/licenses/by-nc-sa/3.0/).

possible length (Crowder et al., 2015; Hara and Kimura, 2009; Wühr et al., 2008). This suggests that the upper limit to the spindle size is set by mechanisms intrinsic to the spindle apparatus, such as the organization of the genome and the specific parameters of microtubule dynamics, and that such mechanisms may be modified to produce scalability in both interspecies and developmental contexts.

Mechanisms of cell-size-dependent spindle scaling

The allotetrapoid frog *Xenopus laevis* is considerably larger than its diploid relative *Xenopus tropicalis*. Fittingly, it is made of larger cells and produces larger eggs with larger nuclei. When egg extracts were used to assemble metaphase spindles around sperm chromatin *in vitro*, the *X. laevis* extracts produced longer spindles, and both cytosolic factors and chromatin contributed to spindle length determination (Brown et al., 2007) (Fig. 3). Higher rates of microtubule disassembly are known to contribute to spindle shortening (Gaetz and Kapoor, 2004; Goshima et al., 2005). Strikingly, *X. laevis* extracts contained considerably lower microtubule destabilization activity because of an Aurora B-dependent inhibitory phosphorylation of the microtubule-severing AAA-ATPase katanin (its p60 subunit), a phosphoregulation modality that is absent in the *X. tropicalis* ortholog (Loughlin et al., 2011; Whitehead et al., 2013) (Fig. 3). Pointing to an evolutionarily conserved mechanism regulating katanin activity through phosphorylation in this region of the protein, phylogenetic analyses reveal consensus phosphorylation sites for a number of mitotic kinases in most orthologs (Whitehead et al., 2013). Variability in potential inputs may fine-tune the katanin-dependent microtubule destabilization in time and space, contributing to spindle scalability in a variety of contexts.

Strikingly, spindle assembly in *X. laevis*, but not *X. tropicalis*, relies on the Ran GTPase gradient to drive microtubule nucleation around the chromatin, and the tetrameric kinesin Eg5 to promote robust antiparallel sorting of interpolar microtubules (Helmke and Heald, 2014) (Fig. 3). This appears to result from a considerably lower cytosolic concentration of the Eg5-interacting microtubule nucleation factor Tpx2 in *X. laevis*, together with attenuation of its microtubule-nucleating activity due to the species-specific 7-amino-acid insertion close to the Eg5-binding site (Helmke and Heald, 2014). In fact, modulating Tpx2 abundance is sufficient to confer the species-specific modes of spindle organization. Tpx2 binds tightly to the negative regulator of spindle assembly importin α, which is far less abundant in *X. tropicalis* than in *X. laevis* (Levy and Heald, 2010). This suggests a model where Tpx2, when present in excess, could indeed stimulate spindle assembly independently of Ran (Helmke and Heald, 2014). Thus, in the case of Tpx2, species-specific differences in relative levels of expression and protein

Box 1. Spindle scaling in the development of a single species

Spindle scaling occurs during early embryonic cleavages in *X. laevis*, where the size of the mitotic spindle decreases with cell size (Wilbur and Heald, 2013). Dependency of spindle assembly on the chromatin-centered Ran-GTP gradient declines with subsequent divisions, with most spindle microtubules nucleated at the centrosomes in smaller cells. In this system, spindle shortening can be accounted for by higher rates of microtubule destabilization due to an increased activity of the microtubule-depolymerizing kinesin Kif2a. Similar to other spindle-assembly factors, Kif2 is inhibited by its interaction with importin α. Importin α exhibits a curious subcellular distribution in frog embryonic cells, partitioning between the cytosol and the plasma membrane, likely due to some form of lipidation. As cell volume falls with each cleavage, the surface-to-volume ratio increases, leading to relative decrease in the cytosolic pool of importin α leaving active Kif2a in the cytoplasm (Wilbur and Heald, 2013).

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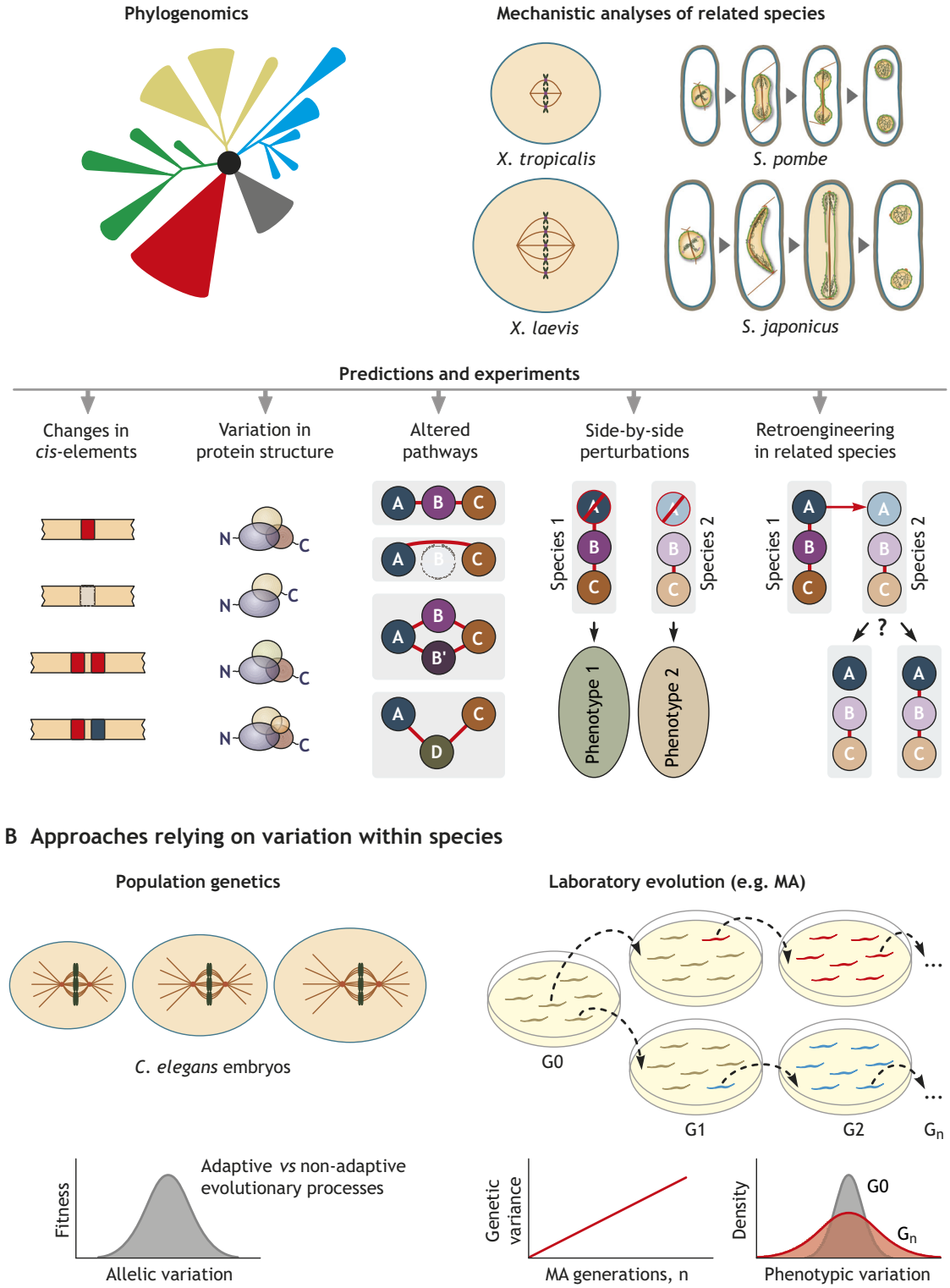

Fig. 4. Experimental approaches used in comparative cell biology. (A) Phylogenomics (indicated by a schematic tree of eukaryotic life) together with mechanistic analyses of closely related species (indicated here as *Xenopus* and *Schizosaccharomyces* pairs of related organisms) can provide novel insights into the rationale of cell biological processes, thereby facilitating discovery of 'new' biology and allowing for retroengineering capabilities. (B) Evolutionary approaches to understanding cell biological variation and genotype-to-phenotype relationship are based on within-species variation. Spindle size variation in *C. elegans* embryos is shown as an example. Population genetics-based methods (left) distinguish between adaptive and non-adaptive evolutionary processes. Laboratory evolution experiments, such as analyses of mutation accumulation (MA) lines (right), eliminate natural selection and may provide insights into the genetic basis of variation in phenotypic traits. MA lines are propagated by bottlenecking, where each new generation (G) arises from a randomly chosen founder, driving spontaneous mutations to fixation (as shown by red and blue worms). The genetic variance between the lines increases with the number of generations (n) and later passages show a considerably increased phenotypic variation.

sequence contribute to distinct specification of spindle architecture. More generally, spindle scaling could likely be achieved through multiple means (Box 1).

Shortening spindles in early blastomers of live *X. laevis* embryos, by decreasing microtubule stability, affects metaphase spindle orientation but does not interfere with chromosome segregation (Wilbur and Heald, 2013). Similarly, lengthening the meiosis I spindles in mouse oocytes leads to the faulty positioning of the cortical cleavage apparatus, without obvious chromosome-partitioning defects (Dumont et al., 2007). Thus, although it remains to be seen whether metaphase spindle size impacts on the fidelity of chromosome segregation, spindle scaling appears to be important for spindle–cortex interactions, which regulate positioning of the cellular division plane in many systems (Oliferenko et al., 2009). Given this function, cell-size-dependent scaling of subcellular structures could be a phenotypic trait subject to selection. Alternatively, it may be an emergent property of self-organization of bio- and mechano-chemical processes.

Probing metaphase spindle size from the evolutionary perspective

Moving from understanding the variance between the cell biological processes to insights into their evolutionary dynamics requires infusing cell biology with evolutionary biology approaches. Evolution occurs at the level of populations and is governed by both adaptive (natural selection) and non-adaptive (mutations, recombination and genetic drift) processes (Lynch, 2007). A particular type of a cell biological process, in a given species, does not necessarily arise as a result of selection for this specific trait but might well emerge due to non-adaptive events. Each evolutionary scenario makes clear assumptions for the distribution of a particular trait within populations, its changes over phylogeny and its relationship to organismal fitness. Unpicking these trajectories requires assessing cell biological variability within different populations of a single species, essentially combining cell biology with population genetics.

A tour de force investigation of one-celled embryos in geographically distinct populations of *C. elegans* and clonal mutation accumulation lines revealed genetic differences underlying distinct spindle dynamics (Farhadifar et al., 2015). However, all measured spindle parameters, including metaphase spindle length, correlated positively with embryo size. As experimentally observed values for embryo size exhibited considerably less variation than predicted by neutral drift, it was concluded that the mutation-stabilizing selection could act to remove individuals deviating from the fitness optimum. *C. elegans* indeed has an optimal embryo size, with embryos deviating from this optimum exhibiting a reduction in fecundity. Importantly, stabilizing selection for embryo size was sufficient to explain variation in all spindle parameters. Thus, at least in this system, the spindle size is not necessarily selected for – rather, it appears to be one of the many traits that correlate with cellular volume. Other nematode species settled on somewhat different embryo size optima but exhibited similar covariance between the spindle and cell size (Farhadifar et al., 2015). These results underscore the importance of intracellular scaling mechanisms that produce spindle variation in response to cell size. However, scaling to cell size likely is not the only reason for variation in spindle architecture found across eukaryotes – after all, spindle parameters may well correlate with other cell biological traits under selection.

Concluding remarks

To conclude, chromosome segregation strategies in eukaryotes have diverged to a remarkable degree. Since chromosome partitioning precedes the division of the cytoplasm, the evolution of mitotic mechanisms is likely intimately associated with that of division plane positioning and cytokinesis. Furthermore, the inherent plasticity of mitosis could be, at least in part, grounded in the functional needs of meiotic divisions that tend to be less well understood even in the best-studied model species. As we are making strides in understanding variability in the molecular mechanisms underlying chromosome segregation, we are learning more about how they intersect with other aspects of cellular physiology.

As illustrated by the examples above, comparative cell biology uses several interrelated approaches. Phylogenomics tackles the genomes, facilitating prediction of gene functions, producing insights into genome dynamics and allowing multi-scale reconstruction of evolutionary relationships. This approach has been extremely useful in providing new insights into the function and evolution of the nucleus and the endomembrane system in eukaryotes, as well as piecing together the possible scenarios underlying eukaryogenesis (Baum and Baum, 2014; Dacks et al., 2016; Devos et al., 2014; López-García et al., 2017; Wilson and Dawson, 2011). Phylogenenomics is therefore useful for predicting a potential for new biology, generating hypotheses and pinpointing interesting models for mechanistic study (Fig. 4A). Mechanistic comparative studies, on the other hand, capitalize on the ‘experiment of Nature’, exploiting apparent diversity in cell biological processes between the related species. This approach with an in-built retroengineering potential identifies the essential elements in the genetic networks underlying a given process and probes how these core relationships can be modified and regulated to produce different phenotypic outcomes (Fig. 4A). Arguably the biggest strength of both sequence-based and experimental comparative studies is providing a wider view of biology without compromising on the depth of insight, by making unanticipated connections between the protein or process of interest and other cellular features.

Understanding the extent of cell biological diversity between species sets the boundary conditions for a given process, which in turn may help constrain rules for modeling the behavior of complex systems both in phylogenesis and ontogenesis. In terms of evolutionary insight, linking variability in cell biological phenotypes to genetic determinants within populations of a single species will be necessary to estimate standing genetic variation and untangle contributions of adaptive versus non-adaptive changes to the phenotype (Fig. 4B). Recent advances in genome sequencing, genetic manipulation and high-throughput imaging have paved the way for merging these threads of inquiry to provide fundamental insights into biology of the cell.

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Competing interests

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