All that is old does not wither: Conservation of outer kinetochore proteins across all eukaryotes?

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The kinetochore drives faithful chromosome segregation in all eukaryotes, yet the underlying machinery is diverse across species. D'Archivio and Wickstead (2017. *J. Cell Biol.* https://doi.org/10.1083/jcb.201608043) apply sensitive homology predictions to identify proteins in kinetoplastids with similarity to canonical outer kinetochore proteins, suggesting some degree of universality in the eukaryotic kinetochore.

High-throughput sequencing technologies combined with comparative genomics have provided insights into the evolution of biological pathways. Computational prediction of homologous pathway components can trace back the ancestral origin of the underlying genes. This approach leverages knowledge of the primary amino acid sequence and is powerful if residues of the protein components are moderately conserved across species. However, its performance is limited when applied to the chromosome segregation pathway, where sequence conservation of several underlying proteins is limited. The lack of sequence conservation among some chromosome segregation components stands in stark contrast to the essentiality of this pathway. Chromosome segregation ensures the faithful transmission of genetic material from generation to generation. Crucial for this process is the kinetochore. The kinetochore is a multiprotein mosaic that assembles onto centromeric DNA to physically couple the movement of spindle microtubules to the separation of sister chromatids during anaphase.

Extensive biochemical and genetic studies in classical eukaryotic model organisms have identified a large catalog of kinetochore proteins (Cheeseman, 2014). Though similar analyses have not been performed in other organisms, computational predictions have identified homologues of several kinetochore proteins in additional species scattered across the tree of eukaryotes (Meraldi et al., 2006; Schleiffer et al., 2012). These findings reveal that most eukaryotic kinetochores consist of at least two common building blocks, namely, the histone H3 variant CenH3/CENP-A at the inner kinetochore and the Ndc80 complex at the outer kinetochore. CenH3 is enriched in centromeric chromatin at the DNA–kinetochore interface and is crucial for the initiation of kinetochore assembly (Howman et al., 2000; Blower and Karpen, 2001; Régnier et al., 2005). The Ndc80 complex binds spindle microtubules at the kinetochore–spindle interface and is crucial for driving sister chromatid separation (Kline-Smith et al., 2005). Given their widespread conservation, it appears unexpected that computational surveys would fail to identify true homologues of canonical kinetochore proteins in kinetoplastids, a group of early-branching protozoans that include the trypanosomes.

In 2014, a pioneering study by Akiyoshi and Gull (2014) performed the first foray into the composition of kinetoplastid kinetochores. These authors applied an elegant candidate approach evaluating chromosomal localization patterns of uncharacterized proteins encoded by cell cycle–regulated genes. This led to the identification of a protein that exhibited a typical "kinetochore-like" localization behavior, termed kinetoplastid kinetochore protein 1 (KKT1). KKT1 was subsequently used as a starting point for iterative protein interaction surveys, which identified 18 additional kinetoplastid kinetochore components. While the KKT proteins are conserved among kinetoplastid species, no detectable homology to canonical kinetochore proteins could be determined, suggesting that kinetoplastids assemble their kinetochores using an alternative set of proteins. In this issue, D'Archivio and Wickstead add to this prior work and identify new kinetoplastid kinetochore proteins, one of which exhibits similarity to canonical outer kinetochore proteins.

D'Archivio and Wickstead (2017) took a reverse approach by applying remote homology predictions targeted for canonical kinetochore proteins followed by experimental validations of predicted candidates in kinetoplastids. Reasoning on a functional constraint for conservation of outer kinetochore proteins (with respect to their essential roles in forming the microtubule interface), the authors undertook a sensitive hidden Markov model (HMM)–based approach to search for remote homologues of the Ndc80 complex, Ndc80 and Nuf2. Both Ndc80 and Nuf2 have similar domain architectures consisting of an N-terminal Calponin homology (CH) fold followed by a C-terminal coiled-coil tail region (DeLuca and Musacchio, 2012). In fact, Ndc80 and Nuf2 are likely derived from a single evolutionary ancestor (Schou et al., 2013). HMM profiles constructed for the two individual protein families, separate or combined into a Ndc80/Nuf2 HMM model, were iteratively matched against proteomes of select eukaryotes. Working from true homologues into more distant evolutionary lineages, these searches identified previously undetected "Ndc80/Nuf2-like" proteins in several organisms; namely, two Excavates and the golden algae *Aureococcus anophagefferens*. Importantly, in organisms with true Ndc80/Nuf2 homologues, no additional

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non-homologous coiled-coil/CH fold proteins were identified, thereby indicating the specificity of the search. Next, HMM profiles containing both true Ndc80/Nuf2 homologues and newly identified hits were matched against profiles of orthologous proteins of select kinetoplastids. This search revealed additional hits with Ndc80/Nuf2-like sequence properties in these organisms. However, sequence similarity to Ndc80/Nuf2 homologues was considerably low and the contribution to detection was from alignment to coiled-coil regions of the profile. Notably, an expected CH domain was not detected in newly identified proteins.

The apparent lack of sequence similarity between canonical Ndc80/Nuf2 proteins and kinetoplastid hits meant that their role at the kinetochore–microtubule interface was still questionable and could not be inferred solely based on their computational predictions. In fact, phylogenetic analyses grouped these newly identified proteins as a separate clade distinct from all known Ndc80 and Nuf2 homologues. Acknowledging this limitation, the authors turned to experimental approaches to evaluate their candidates. As a model system, they chose *Trypanosoma brucei*, the same organism previously used by Akiyoshi and Gull (2014) for the identification and characterization of the 19 KKT proteins. Fluorescently labeling their Ndc80/Nuf2-like candidate allowed D'Archivio and Wickstead (2017) to follow its subcellular localization over the cell cycle. The authors found the localization dynamics to be very similar to KKT1, the first kinetoplastid kinetochore protein identified by Akiyoshi and Gull (2014). D'Archivio and Wickstead (2017) named their newly identified protein KKT-interacting protein 1 (KKIP1).

Further, D'Archivio and Wickstead (2017) examined the functional relevance of KKIP1 for chromosome segregation in *T. brucei*. In vertebrates and fungi, Ndc80 and Nuf2 depletion impairs kinetochore–microtubule binding, leading to aberrant chromosome partitioning and segregation defects (Kline-Smith et al., 2005). Comparably, upon KKIP1 depletion in *T. brucei*, aneuploid cells rapidly accumulated with progressing cell cycles. The authors leveraged the dispensability of *T. brucei* mini-chromosomes for cell viability to further test for chromosome loss in KKIP1-depeleted cells by monitoring the maintenance of marked mini-chromosomes over cell cycles. The authors detected amplified loss rates in the range of one to two orders of magnitude. Overall, their observations are similar to those seen for Ndc80- and Nuf2-compromised cells in other organisms (Kline-Smith et al., 2005). However, D'Archivio and Wickstead (2017) found impaired spindle assembly in KKIP1-depleted *T. brucei* cells—a defect not observed in other organisms. While the mechanistic link is unclear, the authors hypothesize that, in *T. brucei*, spindles are perhaps unstable when not associated with kinetochores.

D'Archivio and Wickstead (2017) next addressed the functional relationship of KKIP1 to the KKT proteins (Akiyoshi and Gull, 2014). The authors performed semiquantitative cross-linking affinity purifications under native, low, and high formaldehyde conditions and mass spectrometry to identify KKIP1 interacting partners. This approach revealed a significant enrichment of several KKT proteins as well as a nuclear pore complex component known to associate with spindles during mitosis. The central mitotic kinase, Aurora B, was also identified, further supporting participation of KKIP1 in the chromosome segregation machinery. Interestingly, the centromere-proximal proteins KKT2 and KKT3, as well as KKT13 that reaches peak levels during S phase (Akiyoshi and Gull, 2014), were not among the potential interaction partners. Collectively with the protein localization studies, these results support a centromere-distal localization of KKIP1, enriched predominantly during mitosis.

In addition, the proteomic analyses identified a new set of potential kinetochore proteins in *T. brucei*. D'Archivio and Wickstead (2017) used the same approaches to characterize the localization and function of these proteins as they did for KKIP1, which allowed them to narrow the list down to six potential interactors, named KKIP2 to 7. While none of these proteins showed any recognizable homologues in species outside the kinetoplastids, KKIP7 was predicted to contain a phosphatase domain belonging to the family that includes members of other known mitotic phosphatases. Correct kinetochore assembly and spindle attachment in other eukaryotes are regulated by the interplay of mitotic kinases and phosphatases that modify proteins of the kinetochore (Reinhardt and Yaffe, 2013). D'Archivio and Wickstead (2017) speculate that KKIP7 acts as an antagonist of trypanosomal mitotic kinases (Aurora B and KKT kinases) to regulate phosphorylation-dependent kinetochore function.

Additional parallels to the outer kinetochore complex could be drawn from insights into the kinetoplastid kinetochore assembly cascade. In other eukaryotes, kinetochore assembly happens in an ordered manner, with the assembly of inner components preceding that of outer ones (Cheeseman, 2014). Consistent with an analogous, centromere-distal arrangement of KKIP1 (and KKIP4), D'Archivio and Wickstead (2017) found that the localization of KKIP1 occurs downstream of most representative KKT members. Conversely, other KKIP proteins were found to be dependent on KKIP1 for recruitment, indicating the upstream localization of KKIP1 in the assembly hierarchy of centromere-distal proteins in kinetoplastids.

To directly observe the arrangement of KKIP1 relative to centromere-proximal proteins such as KKT2, D'Archivio and Wickstead (2017) applied two-color fluorescence microscopy on relaxed kinetochores in anaphase cells. Consistent with its localization closer to the centromere, KKT2 appeared to be significantly skewed away from the spindle pole compared to KKIP1. The calculated distance between the two proteins was similar to the estimated thickness of kinetochore-like plaques observed by electron microscopy in Trypanosomes (Ogbadoyi et al., 2000). Thus, this arrangement of centromere-proximal and -distal proteins recapitulates the size of the *T. brucei* kinetochore complex.

This study by D'Archivio and Wickstead (2017) gives new insights into conserved principles of kinetochore composition and structure. Proteins of the Ndc80 complex are among the most conserved kinetochore components across eukaryotes, yet homologues have not been identified in kinetoplastids. Using bioinformatics analyses and experimental validations, the authors identified a novel kinetoplastid outer kinetochore component with some structural and functional similarity to Ndc80/Nuf2 homologues. Still, it is challenging to provide evidence of homology for several reasons. First, at primary amino acid sequence level, a high degree of sequence divergence is observed. Second, at structural and functional levels, the essential microtubule-binding interfaces found in canonical Ndc80—the N-terminal tail domain, the CH domain, and the characteristic microtubule binding loop region following the CH domain (Varma and Salmon, 2012)—are not detected in the kinetoplastid Ndc80/Nuf2-like candidate, KKIP1. To this end, it is still unclear how kinetoplastid outer kinetochore proteins make

essential microtubule contacts in the absence of otherwise indispensable functional motifs. As far as a universal chromosome segregation model is concerned, the findings from D'Archivio and Wickstead (2017) show that outer kinetochore proteins with recurring structural motifs such as coiled-coil domains are constitutive members of eukaryotic kinetochores (Westermann and Schleiffer, 2013). This thereby indicates some degree of universality of the eukaryotic outer kinetochore complex, particularly with regard to the presence of conserved secondary structures. In addition, this study also proves the potential of D'Archivio and Wickstead's approach for characterizing kinetochore proteins in divergent eukaryotic lineages, which may not have been detected with classical homology searches.

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