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Review

The CCAN complex: Linking centromere specification to control of kinetochore–microtubule dynamics

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ABSTRACT

For over 70 years, chromosomes have been known to oscillate back-and-forth on the metaphase plate. These movements are directed by kinetochores, the microtubule-attachment complexes on centromeres that regulate the dynamics of bound spindle microtubules. Recent evidence shows that the CCAN (Constitutive Centromere Associated Network) kinetochore network, which directly binds centromeric nucleosomes, plays a crucial role in the control of kinetochore microtubule dynamics. Here we review how this 15-subunit protein network functions within the kinetochore machinery, how it may adapt dynamically both in time and in space to the functional requirements necessary for controlled and faithful chromosome movements during cell division, and how this conserved protein network may have evolved in organisms with different cell division machineries.

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1. Introduction

Chromosome segregation acheives faithful partitioning of the duplicated genetic material into the two daughter cells during mitosis. One of the key requirements for successful chromosome segregation is the establishment of a tight metaphase plate, which forms as the chromosomes congress towards the equator of the mitotic spindle [1,2]. Kinetochores attach chromosomes to microtubules and play the central role in chromosome congression by controlling the dynamics of bound microtubules [3–7]. In addition, plus-end directed microtubule-motor forces generated by kinesins also play a role in the initial anti-poleward transport of chromosomes to the spindle equator. Once the metaphase plate is established kinetochore–microtubule dynamics then play the dominant role in controlling chromosome movements [8]. In most eukaryotes, the kinetochore is not bound to a single microtubule, but rather to bundles of microtubules, termed k-fibers, that, depending on the organism, can consist of up to 25 individual microtubules. When the majority of microtubules within a k-fiber undergo catastrophe the k-fiber will shrink. During shrinkage, the kinetochore will remain coupled to the depolymerizing plus-ends, which imparts a pulling force on the chromosomes. If the majority of microtubules within a k-fiber switch to a polymerizing state then the coupled-kinetochore will exert a pushing force on the chromosome. While pushing forces can contribute in certain situations to this process, it is the pulling forces generated at the leading sister-kinetochore are the dominant drivers of poleward movement of the chromosome [9–11].

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In the vast majority of metazoan cells, bipolar-attached chromosomes do not align onto the metaphase plate in a single movement, but rather oscillate back and forth along the spindle axis in regular movements that continue after a chromosome reaches the spindle equator [8,12–14]. The regularity is highly damped and correlations between oscillations do not extend over more than a full period of 75 s. It is unknown whether this emergent oscillatory behavior is simply a reflection of the mechanochemical systems, which underpin directional switching, or whether this regularity is itself physiologically important [15]. In either case, the regularity points to a highly regulated and coordinated control system at the kinetochore, as opposed to purely stochastic microtubule-based dynamic instability [16]. At the molecular level the human CCAN (Constitutive Centromere Associated Network) kinetochore complex was recently identified as a key component of this machinery that directly controls the dynamics of kinetochore–microtubules [17].

2. CCAN – a bridge between centromeric DNA and microtubule plus ends

The CCAN was originally identified as a set of proteins that could be affinity-purified with CENP-A containing centromeric nucleosomes and was, accordingly, originally named CENP-A NAC/CAD (CENP-A Nucleosome Associated Complex/CENP-A Distal) complex or CENP-H/I kinetochore complex (according to its most prominent subunits) [18,19] (Fig. 1). Isolation of the interphase centromere complex also led to the identification of CCAN subunits [20,21]. At the same time a bioinformatic analysis identified many CCAN components as potential orthologues of known kinetochore proteins of the *Schizosaccharomyces pombe* Sim4 complex, suggesting that the CCAN complex is conserved [22,23] (see Table 1 and discussion below). Follow-up studies demonstrated that subunits of this kinetochore complex directly bind to centromeric nucleosomes: the CENP-N subunit binds to nucleosomes containing the centromere-specific histone CENP-A, while the histone-fold proteins CENP-T and CENP-W were shown to bind the adjacent nucleosomes containing the classical Histone H3 [24,25]. CENP-N/-T/-W therefore forms a platform that is required for the hierarchical assembly of the remaining CCAN subunits onto the kinetochore [24]. What is the function of the CCAN? Functional studies indicate that the network is required for the efficient incorporation of CENP-A into centromeric nucleosomes suggesting that the CCAN plays an active role in centromere specification [19,25–27]. CENP-S and CENP-X also contain histone-fold domains [28] and have been shown to associate with the Fanconi anemia core complex [29,30]. This complex has DNA branch migration activity raising the possibility that the CCAN could also be required to maintain centromeric DNA integrity, perhaps during DNA replication in S-phase. Interestingly, in the nematodes *Ceanorhabditis elegans* and *Ceanorhabditis briggsae* CENP-S is fused with an S-methyltransferase (see Table 1).

Once assembled on centromeres the CCAN is thought to be required for the assembly of further kinetochore components thus functioning as a scaffold [7]. This model is supported by experiments showing that the artificial tethering of CENP-T and CENP-C to ectopic chromosomal sites forms a kinetochore capable of recruiting the KMN network and allowing attachment to microtubules [31]. This can be attributed to the direct binding of CENP-T to the Ndc80 complex [31] and CENP-C to the Mis12 complex [61,62]. Previous work had also reported that Ndc80 complex binding to

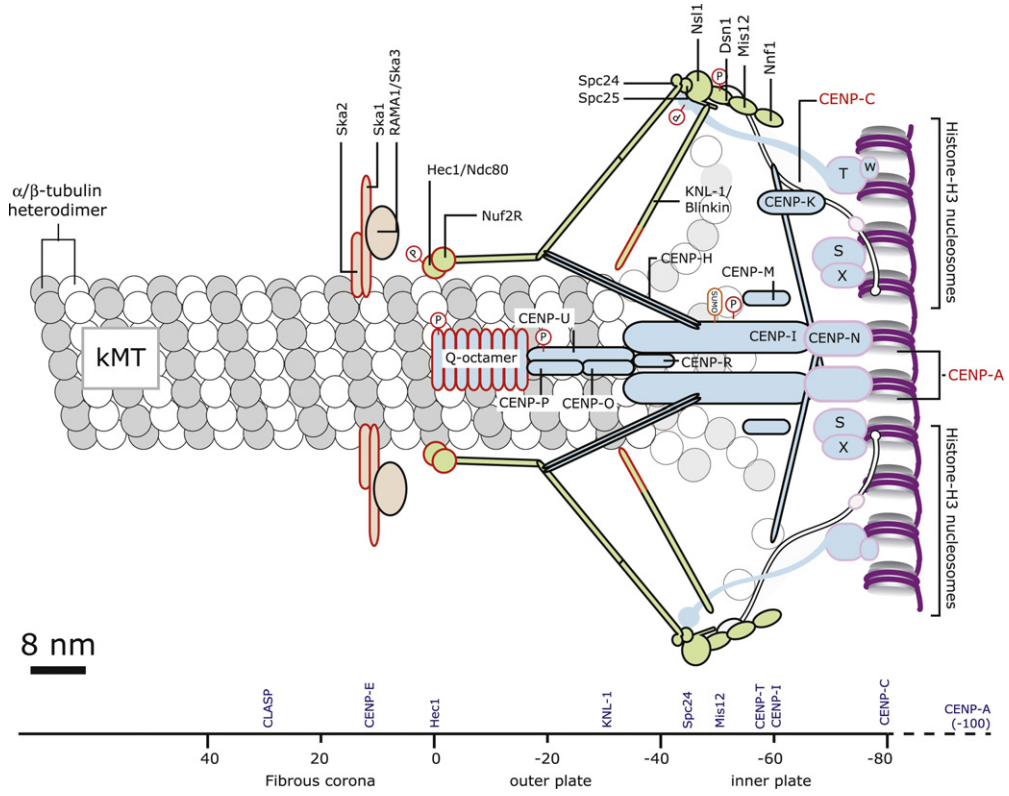

Fig. 1. Protein architecture of a human kinetochore–microtubule attachment site. Subunit composition, post-translational modifications and organization of the KMN network (colored green) and Ska complex (colored orange), which form the core microtubule binding interfaces within the kinetochore, are based on [34,35,60–62]. Subunits of the CCAN, which forms a physical bridge between nucleosomes and microtubules, are colored blue, with those that bind to DNA/histones outlined in pink. Components of the kinetochore that bind directly to microtubules are outlined in red. CENP-C (colored white) acts a linker between CENP-A, the CCAN and the KMN complex. Proteins are drawn to approximate scale based on their predicted molecular weight. Depiction of CCAN components as elongated ellipsoids is based on hydrodynamic measurements where available [17,32]. Post-translational modifications of CCAN proteins were identified in [41,44–49,63,64]. Position of kinetochore components within the kinetochore is based on super-resolution imaging and adapted from [35].

Table 1
Parts list – Constitutive Centromere Associated Network (CCAN).[a]

Name	Synonyms	MW (kDA)	Domain and motifs	*S. pombe*	*S. cerevisiae*	*C. elegans*	*D. melanogaster*	Other organisms
CENP-A	–	15.9	Histone-fold	Cnp1	Cse4	CENP-A	Cid	All eukaryotes
CENP-H	ICEN35	28.5	Coiled coil	Fta3	–	–	–	Unicellular diatoms (XP_002177997)[b]
CENP-I	hMis6, LRPR1	86.7	–	Mis6	Ctf3	–	Bees (XP_001121678)[b]	Slime molds (XP_635116)[b]
							Wasps (XP_001604088)[b]	
							Aphids (XP_001952498)[b]	
CENP-K	Sim4R, Solt, ICEN37	31.7	–	Sim4	–	–	–	–
CENP-L	Fta1R, ICEN33	38.9	–	Fta1	–	–	Wasps (XP_001605964)[b]	Slime molds (XP_635393)[b]
							Aphids (XP_001951878)[b]	Unicellular diatoms (XP_002185884)[b]
CENP-M	ICEN39, PANE1	19.7	–	–	–	–	–	–
CENP-N	Chl4R, BM039, ICEN32	39.6	–	Mis15	Chl4	–	Ants (EFN66598)[b]	–
							Aphids (XP_001945210)[b]	
CENP-O	Mcm21R, ICEN36	33.8	Coiled coil	Mal2	Mcm21	–	–	Plants (AAK25991)
								Slime molds (XP_643970)[b]
CENP-P	–	33.2	Coiled coil	Fta2	–	–	–	–
CENP-Q	–	30.6	Coiled coil	Fta7	–	–	–	–
CENP-R	NRIF3, ITGB3BP	20.2	Coiled coil	–	–	–	–	Restricted to vertebrates
CENP-S	MHF1, FAAP16	15.9	Histone-fold	Mhf1[b]	YOL086W-A[b]	Y48E1C.1[b]	Ticks (XP_002401961)[b]	Plants (XP_002284609)[b]
								Slime molds (XP_638695)[b]
CENP-T	ICEN22	60.4	Histone-fold	SPBC800	–	–	–	–
CENP-U	CENP-50, PBIP1, ICEN24	47.5	–	Fta4	–	–	–	–
CENP-W	CUC2	10.1	Histone-fold	New1	–	–	–	–
CENP-X	D9, Stra13, MHF2	8.9	Histone-fold	Mhf2[b]	YDL160C-A[b]	F35H10.5[b]	Ticks (XP_002403990)[b]	Plants (XP_002889209)[b]

[a] Also known as: CENP-A NAC/CAD [18], CENP-H/I [19] complex, ICEN [20].
[b] All unpublished CCAN orthologues are given with their respective accession numbers or as gene names. For plants the *Arabidopsis thaliana* orthologue is given.

kinetochores was reduced by ~25% following depletion of CENP-H [32] or CENP-K [33]. However, it is important to be aware that depletion of CENP-H, which prevents binding of all CCAN subunits except CENP-T/W/C, does not affect the ability of kinetochore to form stable end-on bipolar attachments [17]. This supports a model in which the CENP-C/-T/-W platform is involved in recruiting the KMN network, whereas the remaining CCAN subunits are not, consistent with the view presented by the Cheeseman and Fukagawa labs in their last study [31].

3. CCAN – a core regulator of kinetochore–microtubule dynamics

This centromeric-centric perspective contrasts with a recent study, which demonstrated that depletion of the CCAN subunit CENP-H eliminates the regularity of chromosome oscillations, increases their speed and causes an increased frequency of directional switches. This suggests a lack of coordination amongst the individual microtubules within a k-fiber and more generally a role for CCAN in the regulation of kinetochore–microtubule dynamics [17]. Consistent with such a hypothesis, CCAN-depleted kinetochores are unable to control tubulin turnover at microtubule plus-ends: wild-type k-fibers have a very low microtubule plus-end turnover, while k-fibers of cells lacking the CCAN complex have a turnover that is 20-fold higher, equivalent to free spindle microtubules [17]. Importantly, the regulation of kinetochore microtubule-dynamics by CCAN is most likely direct: one of its subunits, CENP-Q, forms a homo-octamer that can bind microtubules *in vitro* (with a K_d similar to that of the Ndc80 complex; [34]) and the CCAN complex itself is known to co-localize with the microtubule-plus ends in the kinetochore structure [17,35].

One of the main functions of the CCAN network appears to be the reduction of kinetochore–microtubule plus-end turnover, *i.e.*, to prevent an uncontrolled and rapid dynamic instability of kinetochore–microtubules. Loss of this controlled plus-end turnover could stop other regulators of microtubules at kinetochores, such as the microtubule-depolymerases Kif18A and MCAK [8,36], from affecting chromosome oscillations, as their effect would be diluted out in sea of very rapid dynamic instability. This would explain the rapid directional switches of metaphase chromosomes seen in CENP-H depleted cells [17]. In the absence of a more detailed molecular mechanism, it is unclear whether the reduced plus-end turnover explains the regularity (and memory) of the chromosome oscillations, or whether this effect is due to the additional ability of CCAN to coordinate the growth and shrinkage of different kinetochore microtubules in a single kinetochore fiber. A crucial questions for the future will be to unravel the exact mechanism by which the CCAN network affects microtubule dynamics and suppresses the high turnover of mitotic microtubules. Such insights are most likely to come from *in vitro* studies with reconstituted CCAN (sub) complexes and purified microtubules. A second key question is why the CCAN complex consists of 15 different subunits, and what might be the respective functions of the different subunits.

4. The CCAN network is highly dynamic and can specifically recognize growing microtubules

The CCAN, as its name suggests, is thought to remain bound to the centromeres throughout the cell cycle. This view is rooted in the experiments that initially identified CENP-H and CENP-I and showed that they remained bound to centromeres during both mitosis and interphase [38,39]. Photobleaching experiments confirm that CENP-H and CENP-I are stably bound to kinetochores [40]. However, recent evidence supports an alternative view of the

CCAN as a highly dynamic network of proteins that are cell cycle regulated and responsive to the mechano-structural state of the kinetochore. While CENP-H and -I are constitutively bound, other subunits of the CCAN are not. CENP-U is absent from anaphase kinetochores because it is targeted for degradation in a polo-like kinase dependent manner [41]. Moreover, kinetochore binding by CENP-O and CENP-N is reduced by 40% and 80%, respectively in metaphase compared to interphase [42,32]. The reduction in CENP-N binding is particularly interesting given its function as the CENP-A-nucleosome binding subunit of the CCAN (see above); are CENP-N nucleosome binding events only required during an earlier (pre-mitotic) step during kinetochore assembly? Do kinetochores need to "loosen" their grip on the chromosome during/before anaphase? The former idea is supported by experiments in budding yeast, which show that the CENP-N orthologue Chl4p is required for de novo kinetochore formation [43]. Although the CENP-H/-I subunits of the CCAN are present on kinetochores throughout the cell cycle, recent work has revealed that they are asymmetrically localized on the two sister kinetochores – with these subunits enriched on the trailing (anti-poleward moving) sister [17] (Reproduce figure here?). This surprising result shows that the CCAN can differentiate between two structural and mechanical states of the kinetochore: one sister that is attached to growing microtubules and the other sister that is attached to shrinking microtubules. The function and reason for this asymmetry is discussed below, but it clearly demonstrates that the CCAN forms a key adaptive part within the kinetochore machinery and that it is not simply a structural scaffold. This finding is consistent with previous work showing functional flexibility with the CCAN complex. Indeed, epistasis experiments based on single and double siRNA depletions indicate two functional layers controlling microtubule dynamics with CCAN: a basic layer containing CENP-H, -I and CENP-K, which decrease the stability of microtubules and which is counterbalanced by a second layers consisting of CENP-O, (and most likely CENP-U, -Q, -P and -R), which stabilize kinetochore–microtubule stability (Fig. 1) [32]. Interestingly, depletion of CENP-O which leads to a reduction of microtubules within the k-fiber and a failure to assemble a bipolar spindle, also leads to an increase in the levels of CENP-H and -I, strengthening the view that the CCAN complex reacts and adapts to different mechanochemical status of the microtubules [11,32]. Thus our current working model envisages the CCAN as an adaptive molecular machine that responds to and controls tubulin exchange at the plus-end of spindle microtubules.

5. What controls the asymmetry of CCAN proteins?

A crucial future question is to know the molecular mechanisms that control the asymmetry of the CCAN proteins. The first point is that it is not the number of CCAN complexes at kinetochores that changes but the subunit stoichiometry within the complex. Indeed, while CENP-H and CENP-I are asymmetric, the CENP-O and CENP-P subunits are symmetric [17]. Any mechanism must therefore be based on the selective loading or unloading of specific subunits into the complex. Post-translational modifications that modulate binding of these subunits are prime candidates, in particular protein phosphorylation. Potential upstream candidates would include all the proteins kinases that are known to bind kinetochores and regulate kinetochore microtubule attachment, such as Aurora B, Plk1, BubR1, Bub1 and Mps1 [2,5]. Consistent with this possibility multiple mitotic phosphorylation sites have been reported for CCAN subunits [41,44–48]. In particular, the CENP-U (also known as PBIP1) subunit is heavily phosphorylated during mitosis by Plk1 [41]. More recently, this CENP-U-Plk1 complex has been shown to bind CENP-Q leading to its phophorylation [44]. It is also possible that other modifications are involved: for example CENP-I is sumolyated during mitosis [49]. Another intriguing possibility, however, is that the asymmetry of the CCAN complex is not based on protein modification, but on the oligomerization kinetics of CCAN subunits – perhaps in direct response to whether microtubules in the k-fiber are in a growth or shrinkage phase. Indeed, it is known that in the fission yeast *S. pombe* kinetochores bind 4 copies of the CENP-H and CENP-K orthologues per microtubule [50]. Moreover, several of those proteins are capable of interacting with themselves in two-hybrid assays, exogenous CENP-H can interact with itself at kinetochores in cells, and our biochemical work shows that recombinant CENP-Q forms a homo-octamer [17,32,51]. CENP-Q has been shown to also interact with itself in cells supporting the formation of octamers *in vivo* [44]. Interestingly this self-association is required for kinetochore-binding [44]. It is therefore possible to imagine that growing microtubule plus-ends do not have to necessarily activate a signaling cascade that would lead to an accumulation of CENP-H and CENP-I, but instead affect the oligomerization capacity of such proteins, resulting in different stoichiometries within the CCAN complex.

A second key aspect is whether the asymmetry of CCAN proteins is a cause or consequence of changes in microtubule plus-end dynamics at sister kinetochores and what the function of the complex under these two situations could be. A downstream function (CCAN composition adapts following a shift in microtubule dynamics; Fig. 2, model 1) would mean that the accumulation of CENP-H and CENP-I-class subunits is triggered following a directional switch when microtubules are rescued into a growth phase. This would be similar to what is likely to be happening in the case of EB1's accumulation to the trailing sister [37]. It will be interesting to determine whether any CCAN subunits, like EB1, are able to track the growing end of a microtubule *in vitro* [53]. The accumulating proteins would then function to damp the turnover of tubulin at the plus-ends – perhaps by suppressing catastrophe events – and therefore maintaining "processive" movement of the sister pair polewards. As well as directly regulating microtubule dynamics (perhaps via CENP-Q) the asymmetry of CCAN could be critical in to allowing the kinetochore-bound microtubule depolymerases, such as MCAK or Kif18A, to modulate oscillations at either the leading of trailing sister. A strong prediction would be that loss of such mechanisms would cause more frequent directional switches during kinetochore oscillations. Alternatively, an upstream function (a shift in CCAN composition produces a shift in microtubule dynamics; Fig. 2, model 2) would require that CCAN subunits would accumulate on the trailing sister kinetochore prior to the directional switch. This accumulation could reach a threshold, which triggers the directional switch event – perhaps by causing catastrophe of microtubules in a coordinated fashion, thus initiating k-fiber depolymerization. In this case the accumulation of CENP-H or CENP-I on the trailing kinetochore would act as an intrinsic oscillation timer, reflecting the progress of protein modification or alternatively of the kinetics of oligomerization of CCAN proteins. In this regard it is very interesting that a recent mathematical model of chromosome directional instability involves a mechanochemical feedback mechanism that assumes the presence of an unknown regulator at the kinetochore, which reaches a given threshold and promotes microtubule growth [54]. Whether the accumulation of CENP-I and CENP-H reflects part of such a mechanochemical system will require further mathematical and experimental work.

Model 2 would predict longer times between directional switches in the absence of the CCAN asymmetry. Our current data following complete loss of the CCAN would support the opposite model [17]. However, the specific consequence of losing only asymmetry, while leaving the complex intact, is unknown. Another criteria that would discriminate between these two models is the level of GFP-CENP-I on sisters undergoing directional switches at high temporal resolution. If GFP-CENP-I begins to accumulate after

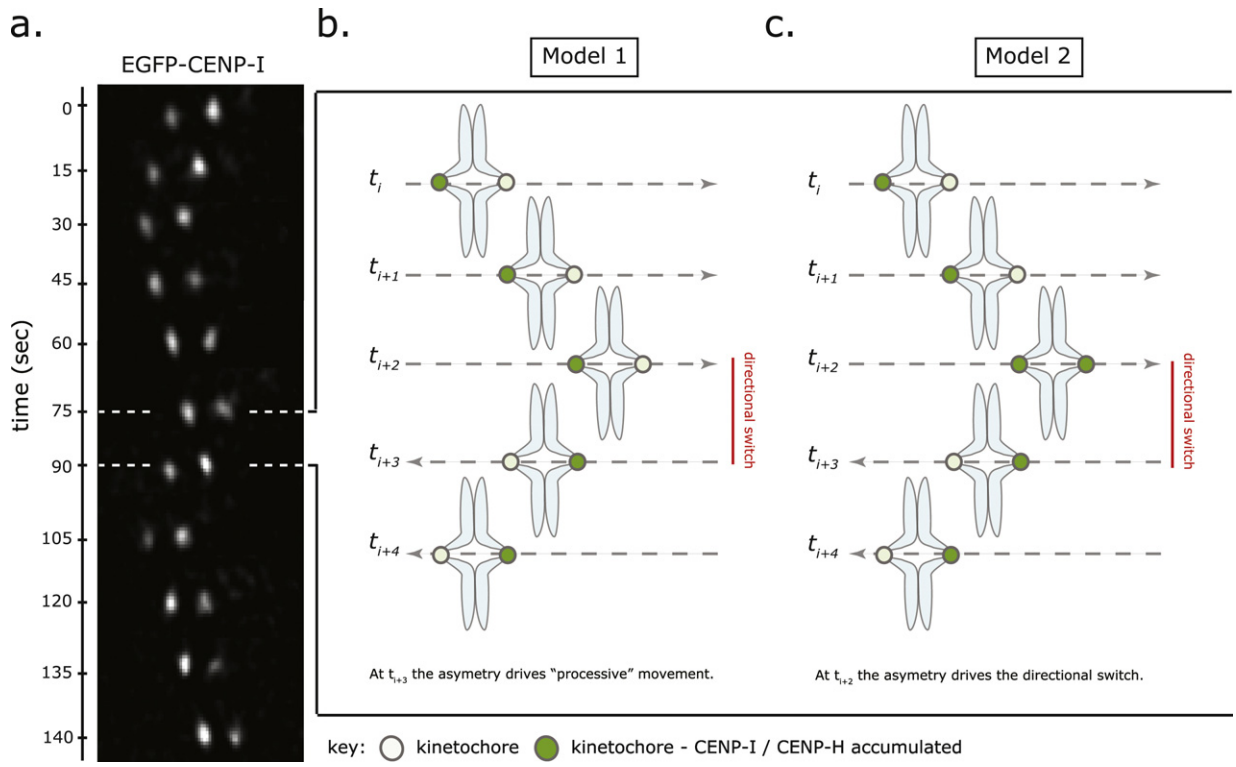

Fig. 2. CENP-I is asymmetrically localized on sister kinetochores. (a) The CENP-A NAC/CAD subunit CENP-I preferentially accumulates on the trailing sister kinetochore. An example time-lapse (1 frame/15 s) sequence from a HeLa stable cell line expressing EGFP-CENP-I is shown. The sister pair undergoes 3 directional switches between T = 30–45 s, 75–90 s and 105–120 s. In each case CENP-I is enriched on the trailing sister kinetochore. (b) Two models to explain the order-of-events during a directional switch (*e.g.*, between 75 s and 90 s). Model 1 suggests that CENP-I accumulates after the directional switch and the asymmetry at this time point (t_{i+3}) is important for maintaining "processive" movement of the sister pair. Model 2 suggests that the CENP-I is accumulating prior to the directional switch (t_{i+2}) and that the asymmetry at this point is involved in triggering the switch.

the directional switch then this would favor model 1, whereas an accumulation preceding the directional switch would favor model 2. In either case, careful *in vitro* experiments at high temporal resolution will be essential to determine how CCAN subunits directly regulate microtubule dynamic instability. This will not be an easy approach since CCAN may only function in the context of multiple parallel microtubules and moreover, multiple subunits may be required to reconstitute the *in vivo* effects on microtubule dynamic instability.

6. Is the CCAN complex conserved? What is the evidence that its function is conserved?

One final issue is whether the CCAN machinery is conserved in terms of subunit composition and function throughout evolution. At first sight, one could conclude that this complex might only be working in vertebrates, as many or even all equivalent components cannot be found in the classical model organisms *Saccharomyces cerevisiae*, *C. elegans* and *Drosophila melanogaster*. However, a more detailed analysis reveals a more differentiated view. Nearly all human CCAN proteins are conserved in the fission yeast *S. pombe* (CENP-R is the exception as it is only found in vertebrates) and several components such as CENP-H, -I, -L, -O, -S, or -X can be found in more distantly related plants, slime molds or unicellular diatoms ([23,32,55] and Table 1). This strongly suggests that the CCAN complex, like the extremely well conserved KMN kinetochore network, is ubiquitously present in all kingdoms of eukaryotes. But then why have these proteins not been found in *S. cerevisiae*, *C. elegans* and *D. melanogaster*? In the case of the *D. melanogaster* we and others have previously found that the sequence of fly kinetochore proteins have rapidly diverged in the course of evolution, raising the possibility that these proteins are present in flies, but cannot be recognized at the sequence level [23,56]. Consistent with such a hypothesis, several components (CENP-L, -I, -N, -S and -X) are present in bees, wasps or ticks, strongly suggesting that this complex has not been lost in insects (Table 1). The situation is different in the budding yeast, *S. cerevisiae*, and possibly in nematodes, such as *C. elegans*. *S. cerevisiae* indeed does not posses a regional centromere, like the vast majority of eukaryotes, but rather relies on sequence-specific point centromeres, that in turn support kinetochores that only bind a single microtubule [57]. Such a configuration has emerged in a small subset of budding yeasts, and it is striking that all these organisms have lost over 50% of the CCAN proteins and replaced them with another set of budding yeast specific proteins [23]. As there is no need for coordination of microtubules within k-fibers, one plausible explanation could be that this particular function has been lost along a specific set of CCAN proteins. Along the same lines, it is possible that nematodes, which rely on holocentric kinetochores that cover the wholes chromosomes, lost most CCAN proteins (although we note the presence of the CENP-S/-X-complex, which might only work in concert with the Fanconi anemia core complex; Table 1) for a similar reason: these organisms do not need to coordinate single microtubules within a k-fiber, but rather have to coordinate many more microtubules all along the mitotic spindle, a behavior that is still very poorly understood till date.

7. Future view

The CCAN forms a core conserved part of the kinetochore that has the unique ability to form a direct physical bridge between the centromeric nucleosomes and the microtubules. It is tempting to imagine that such a structure could reflect an ancestral

kinetochore, which was built on a regional centromere with a minimal DNA and microtubule-binding capacity [23]. As centromeres evolved the CCAN would have rapidly diverged and adapted to its microenvironment. We speculate that the capacity to form a nucleosome–microtubule bridge provides a mechanism to center a microtubule-binding site on CENP-A-nucleosomes. This way a centromere would be able to assemble and coordinate multiple correctly spaced, microtubule attachment sites. This model is consistent with super-resolution imaging that shows CENP-H localizing within CENP-A rich subdomains and excluded from H3-domains. The model is also consistent with electron-microscopy data, which shows that the microtubule-tip is very precisely centered above the centromeric nucleosome(s) [58,59]. The limited conservation of the CCAN in budding yeast and worms may simply reflect the presence of a single attachment site or distributed attachment sites across the chromosome respectively. Once centered the KMN and Ska complexes would then contribute the core, load-bearing, microtubule binding activity within the kinetochore, with the CCAN functioning to control the exchange of tubulin heterodimers at the microtubule plus-end.

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