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***Drosophila* CLASP is required for the incorporation of microtubule subunits into fluxing kinetochore fibres**

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Abstract

The motion of a chromosome during mitosis is mediated by a bundle of microtubules, termed a kinetochore fibre (K-fibre), which connects the kinetochore of the chromosome to a spindle pole. Once formed, mature K-fibres maintain a steady state length because the continuous addition of microtubule subunits onto microtubule plus ends at the kinetochore is balanced by their removal at their minus ends within the pole. This condition is known as ‘microtubule poleward flux’¹. Chromosome motion and changes in position are then driven by changes in K-fibre length, which in turn are controlled by changes in the rates at which microtubule subunits are added at the kinetochore and/or removed from the pole². A key to understanding the role of flux in mitosis is to identify the molecular factors that drive it. Here we use *Drosophila melanogaster* S2 cells expressing α -tubulin tagged with green fluorescent protein, RNA interference, laser microsurgery and photobleaching to show that the kinetochore protein MAST/Orbit — the single CLASP orthologue in *Drosophila* — is an essential component for microtubule subunit incorporation into fluxing K-fibres.

CLASPs belong to an expanding class of proteins that track along the plus ends of growing microtubules (called +TIPs). They are thought to be involved in regulating microtubule dynamics during both interphase and mitosis³. In marked contrast with other +TIPs, CLASPs are unique because they bind to kinetochores throughout mitosis in the absence or presence of attached microtubules^{4,5}. We have previously shown that inhibiting CLASP function induces spindles to collapse and causes abnormal chromosome congression, suggesting that CLASP is involved in regulating microtubule dynamics, particularly within K-fibres^{5,6}.

To examine this issue in detail, we depleted MAST/Orbit in *Drosophila* S2 cells stably expressing green fluorescent protein (GFP)-tagged α -tubulin (S2T cells⁷) by RNA interference (RNAi). This system has several advantages for such studies. First, MAST/Orbit is the only *Drosophila* orthologue of CLASPs, which eliminates possible redundant functions between CLASP1 and 2. Henceforth, the term ‘CLASP’ will be used when referring to MAST/Orbit. Second, K-fibres in live S2T cells are robust and can be easily discerned (Fig. 1a). Also, spindle formation has been characterized in detail in these cells⁸. Importantly, in S2T cells the formation of K-fibres is often initiated by kinetochores. As a result, it is not unusual to find K-fibres that formed from the kinetochore without a relationship to an existing centrosome/aster

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(see Supplementary Information, Movie 1a)⁸. Together these features provide a unique opportunity for studying how depleting CLASP affects the formation and maintenance of K-fibres during mitosis in animals.

After 72 h of RNAi treatment, S2T cells are depleted of CLASP (> 95%; Fig. 1b)⁶. As in controls, in the absence of CLASP centrosomes were often positioned on opposite sides of the nucleus before nuclear envelope breakdown (NEB; Figs 1a, c). After NEB, K-fibres rapidly formed on chromosomes positioned between the separated centrosomes (compare Fig. 1a with c). However, unlike in controls, once formed in CLASP-depleted cells the spindle and its associated K-fibres gradually and progressively shortened (Fig. 1c; also see Supplementary Information, Movie 1b). This confirms earlier conclusions that interfering with CLASP induces bipolar spindles to collapse, providing direct evidence for the shortening of K-fibres during this process. Interestingly, the initial rate of spindle collapse in CLASP-depleted cells was about $1.2 \mu\text{m min}^{-1}$, or $\sim 0.6 \mu\text{m min}^{-1}$ for each half spindle ($n = 5$; Fig. 1e and Table 1). However, this rate was not linear with time, implying the presence of a resistant force to spindle shortening. This resistance could arise from varying degrees of CLASP depletion, which could lead to heterogeneity in the severity of the phenotype. However, there is good evidence (for example, see ref. ⁹) that both microtubule motor molecules and the intrinsic dynamic properties of microtubules are required to maintain spindle bipolarity. Thus, a variable resistance to collapse after CLASP RNAi more likely arises from varying structure of forming spindles combined with different activities of motor proteins such as KLP61F/Eg5, members of the Kinesin-5 family¹⁰, which work to maintain spindle bipolarity. This scenario is supported by the observation that spindle collapse in yeast STU1 mutants (the *Saccharomyces cerevisiae* CLASP) can be rescued by doubling the dosage of Cin8p, the counterpart of KLP61F/Eg5 in this organism¹¹.

Our initial observations confirmed that CLASP activity is not required for the genesis of K-fibres. In fact, it was not unusual in CLASP-depleted cells to see K-fibres form, as described above for controls, by a mechanism intrinsic to kinetochores⁸ (Fig. 1c). However, as spindles collapsed in CLASP-depleted cells, mature K-fibres not connected to a spindle pole shrank as well (Fig. 1d). This not only reveals that CLASP becomes essential for maintaining K-fibres after they reach a particular maturation stage, but also that K-fibre shortening in cells lacking CLASP occurs by a mechanism intrinsic to the kinetochore or K-fibre and not the spindle poles. In this regard, we recently postulated that CLASPs regulate the dynamics of K-fibres by mediating microtubule subunit incorporation at the kinetochore⁵. If this is true, mature K-fibres in CLASP-depleted cells should not exhibit microtubule subunit flux¹².

To directly test this prediction, we photobleached short stretches of individual K-fibres in control and CLASP-depleted S2T cells. In control metaphase cells, photobleached marks moved poleward at a rate of $0.70 \pm 0.15 \mu\text{m min}^{-1}$ ($n = 5$; Fig. 2a, e and Table 1). However, in CLASP-depleted cells the bleached mark remained stationary as the spindle gradually collapsed (Fig. 2b, e). CLASPs are components of the kinetochore corona and are the last proteins known to become incorporated into this structure^{5,13}. As a result, depleting CLASPs does not affect the targeting of other kinetochore proteins, including CENP-A, CENP-B, CENP-C, CENP-E, Polo, Zw10, CLIP-170 and dynein^{5,6}. Thus, it is likely that the absence of microtubule subunit flux in CLASP-depleted cells is due to the direct involvement of CLASP.

To confirm that the effects observed in CLASP-depleted cells are not due to a general perturbation of the kinetochore–microtubule interface, we repeated our photobleaching experiments in cells depleted of either cytoplasmic dynein — another corona protein¹⁴ — or EB1, which is also a tracking protein delivered to the kinetochore on elongating microtubules¹⁵. As previously reported, when dynein heavy chain is knocked down, S2T cells

still form bipolar spindles with properly aligned chromosomes that are delayed in metaphase^{7,8}. However, as in plants, the spindle poles in dynein-depleted S2T cells were frequently splayed (Fig. 2c; also see ref. ⁸). As reported by others¹⁶, we also found that depleting EB1 in S2T cells led to a severe reduction of astral microtubules and bipolar spindles that were shorter than those of controls (compare Figs 2a and d). Importantly, however, photobleached marks on K-fibres in both dynein- or EB1-depleted cells exhibited persistent poleward motion with rates similar to controls (Fig. 2a–e and Table 1). Together, these results provide compelling evidence that the lack of microtubule subunit flux in CLASP-depleted cells is due specifically to the depletion of CLASP. This, in turn, means that CLASP is required for microtubule subunit addition into mature K-fibres, and in its absence spindles collapse because K-fibres progressively shorten by microtubule subunit loss at their minus ends. Here it is noteworthy that a similar phenotype occurs when vertebrate metaphase cells are treated with low doses of Taxol, which similarly inhibits subunit incorporation into kinetochore microtubule plus ends, but not their removal at the pole¹⁷. Nevertheless, as CLASP family members are also integral centrosome components^{4,5}, it is possible that CLASP depletion also affects microtubule minus-end dynamics.

To examine this possibility, and to also directly determine the role of CLASP at kinetochores, we severed the connection between kinetochores and spindle poles in S2T cells by cutting mature K-fibres with a laser. As shown previously by us⁸ (and by others using different systems^{18,19,20}), when K-fibres in control cells were severed, the fragment that remained connected to the pole (the P-fragment) rapidly disassembled towards the pole at $21.80 \pm 0.35 \mu\text{m min}^{-1}$. In contrast, the fragment that remained attached to the kinetochore (the K-fragment) re-grew to its original length at $0.78 \pm 0.17 \mu\text{m min}^{-1}$ (Fig. 3a and Table 1; also see Supplementary Information, Movie 2a)⁸.

As in control cells, when mature K-fibres were severed in CLASP-depleted cells, the P-fragments rapidly disassembled towards the spindle pole (Fig. 3b and Table 1; also see Supplementary Information, Movie 2b). Thus, CLASP depletion does not modify the dynamics of free K-fibre microtubule plus ends created by laser microsurgery. However, in marked contrast to controls, in all 30 CLASP-depleted cells examined, the K-fragments did not change length (that is, re-grow) during the course of the experiment, even as the spindle gradually collapsed (Fig. 3b).

We have previously shown that severed K-fibre re-growth in control S2T cells occurs because the new microtubule minus ends remain stable, whereas microtubule subunits continue to be incorporated into kinetochore microtubule plus ends⁸. Therefore, in control or CLASP-depleted cells the machinery for removing subunits from microtubule minus ends on K-fragments must not be present. As a result, depleting CLASP has no discernible impact on microtubule minus-end dynamics. Accordingly, when we repeated these severing experiments in dynein- or EB1-depleted cells, the results were indistinguishable from the behaviour of K-fragments in control cells (Fig. 3a–e and Table 1, also see Supplementary Information, Movies 2c, d). Together, these results reveal that K-fragments in CLASP-depleted cells do not elongate, and bleach marks do not move poleward, because CLASP is required for kinetochore-mediated microtubule subunit incorporation into mature (fluxing) K-fibres (Fig. 4). This conclusion is consistent with the observation that CLASP binds to tubulin subunits *in vitro* (S. Gouveia, P. Sampaio, R. Reis and C. E. Sunkel, unpublished observations).

In *Drosophila* and most probably other systems²¹, K-fibres that form from the kinetochore do so by a two-step process. First, short microtubule stubs associated with the kinetochore corona begin to elongate by subunit addition at their kinetochore-attached plus ends. This continues until the fibre reaches its mature length, at which time this steady state length is maintained until the onset of anaphase. Both of these processes require subunit addition into microtubule

plus ends at the kinetochore. However, our data reveal that these two processes occur by different mechanisms. Because K-fibres in CLASP-depleted S2T cells still form by microtubule elongation from the kinetochore, this process is independent of CLASP. In contrast, the mechanism that maintains the length of the K-fibre after it forms, which is based on microtubule subunit flux, clearly involves CLASP. This means that the properties of forming and mature K-fibres differ. In this regard it is possible that CLASP becomes important for kinetochore microtubule dynamics only after the flux machinery becomes engaged. For example, the switch between CLASP-independent K-fibre formation and CLASP-dependent K-fibre maintenance may occur as the molecules responsible for removing subunits accumulate at microtubule minus ends and become operative.

KLP10A, a member of the Kinesin-13 family, has been implicated in K-fibre shortening during mitosis in *Drosophila* embryos by mediating removal of microtubule subunits at the poles². Its human orthologue, Kif2a, is also required for spindle bipolarity⁹. A molecular view is therefore emerging for how K-fibres elongate and shorten to allow for chromosome motion and position changes in mature spindles: CLASP is involved in regulating microtubule subunit incorporation into K-fibres at the kinetochore, whereas proteins such as KLP10A/Kif2a regulate microtubule depolymerization at the poles (Fig. 4). Any imbalance in the activity of these proteins affects K-fibre length. This leads to changes in the position of the chromosomes relative to the poles and spindle equator, while also establishing spindle length. In crane flies and grasshopper spermatocytes, the shortening of K-fibres occurs exclusively by enhancing the rate of subunit removal near the spindle pole without dampening the rate of incorporation at the kinetochore^{22,23}. However, in vertebrates and *Drosophila*, K-fibre shortening occurs primarily by dampening microtubule subunit incorporation at the kinetochore without changing the rate of subunit removal at the pole^{24–26}. Our data suggest that in these latter systems, switches between K-fibre shortening and elongation, required for poleward and away-from-the-pole kinetochore motion^{27,28}, are regulated by changes in the functional state of CLASPs. Finally, we found that mature K-fibres not connected to a spindle pole shorten in CLASP-depleted cells. This implies that the stable microtubule minus ends of a growing K-fibre in S2T cells switch, at some point in their maturation, into a depolymerization state independent of their association with a centrosome. This most probably occurs as the minus ends recruit molecular factors involved in microtubule subunit removal.

METHODS

Growing and flattening S2T cells for laser microsurgery and photobleaching

Drosophila S2T cells were a gift from R. Vale (University of California, San Francisco, CA). The cells were flattened for live cell microscopy analyses, and laser microsurgery/photobleaching studies were conducted as described⁸. Time-lapse images were collected at 24–26 °C using either an Olympus IX70 or a Nikon Eclipse TE2000E DIC inverted light microscope equipped with CM350 or CoolSnap HQ cameras, respectively, (Photometrics, Tucson, AZ). Time-lapse data sets were subsequently deblurred using the Softworx 2.5 deconvolution algorithm (Applied Precision, Issaquah, WA). Image sequences were compiled with ImageJ 1.30 (National Institutes of Health) and processed for publication using Adobe Photoshop 6.0.

RNAi

RNAi depletion of *Drosophila* CLASP, dynein and EB1 were conducted as described²⁹ using published targeting sequences^{6,8,16}. Cells were analysed by light microscopy or by immunoblots three days after adding dsRNA to cultures, except for *EB1* RNAi, where efficient depletion could only be accomplished after a second treatment with fresh dsRNA for another three days (see Supplementary Information, Fig. S1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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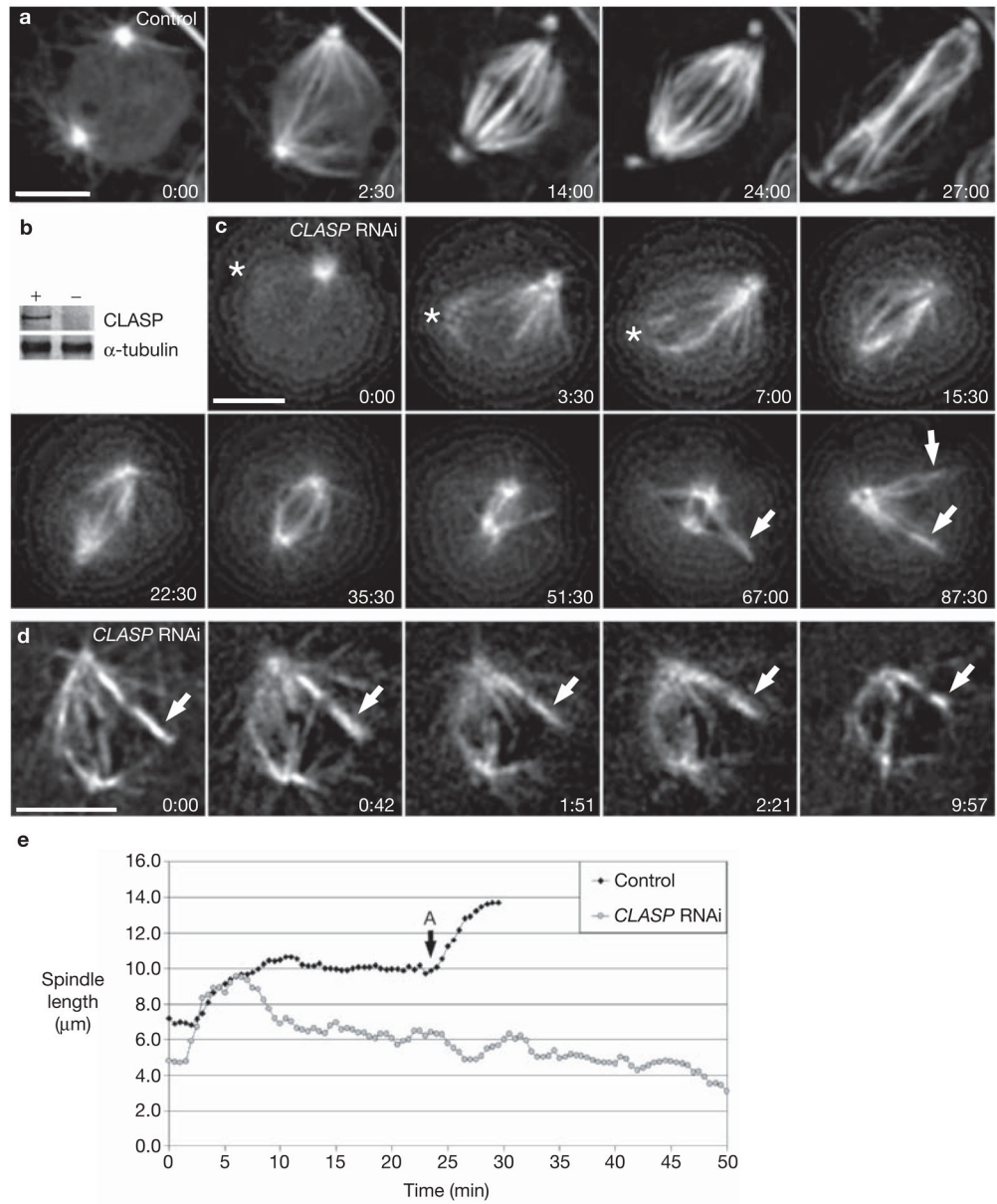


Figure 1. CLASP is required for maintaining spindle and kinetochore fibre length in *Drosophila* S2T cells. **(a)** Mitotic progression in a control cell stably expressing GFP- α -tubulin. **(b)** Western blot showing the depletion of *Drosophila* CLASP by RNAi (–) compared with control S2T cells (+). Endogenous α -tubulin levels are shown as a loading control of protein extracts derived from 10^6 cells per lane. **(c)** Spindle collapse in a *Drosophila* S2T cell depleted of CLASP by RNAi. Note that several kinetochore fibres were generated by kinetochores (arrows) as the spindle collapsed. Asterisks indicate a centrosome not visible in some focal planes during the early recording stages. **(d)** Mature K-fibres, not attached to a centrosome/spindle pole (arrow), also shorten as the spindle collapses in cells depleted of CLASP. Time is shown in min:sec; scale bar represents 5 μ m. **(e)** Spindle length over time for the control **(a)** and CLASP-RNAi-depleted **(c)** cells. ‘A’ indicates the onset of anaphase in the control cell in the chart. Movies of **a** and **c** can be found in the Supplementary Information.

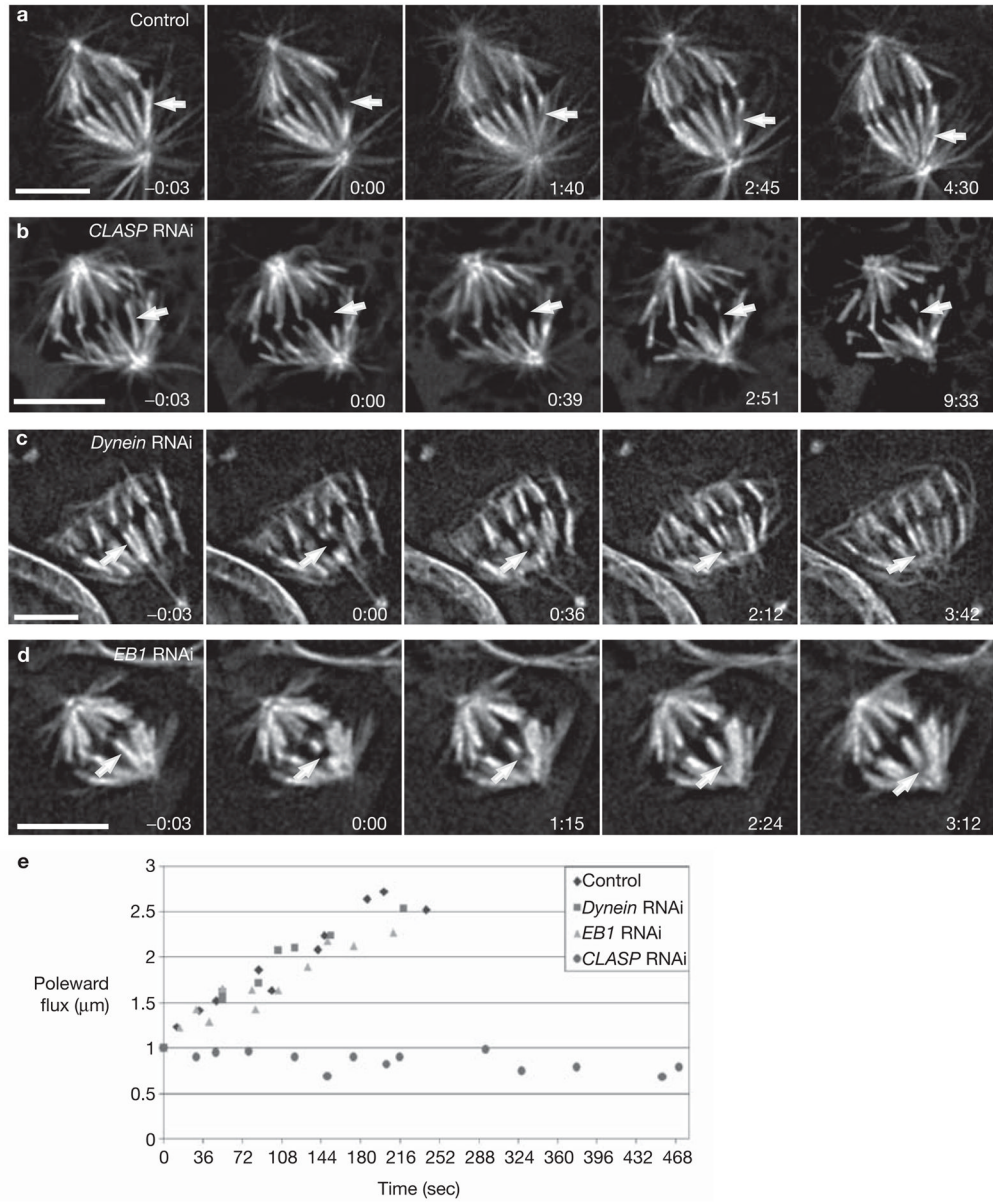


Figure 2. CLASP is required for microtubule poleward flux. **(a)** Control cell showing the poleward motion (microtubule subunit flux) of a photobleached region (arrow) on a K-fibre. **(b)** In cells depleted of CLASP, photobleached marks on K-fibres (arrow) do not move poleward as the spindle gradually collapses. **(c, d)** Photobleached regions on K-fibres in dynein- or EB1-depleted cells, respectively, flux poleward (arrow) at normal rates. Time is shown in min:sec; scale bar represents 5 μm . **(e)** Kinetic traces of poleward flux, as assayed from the motion of photobleached regions on K-fibres, from two different cells per condition.

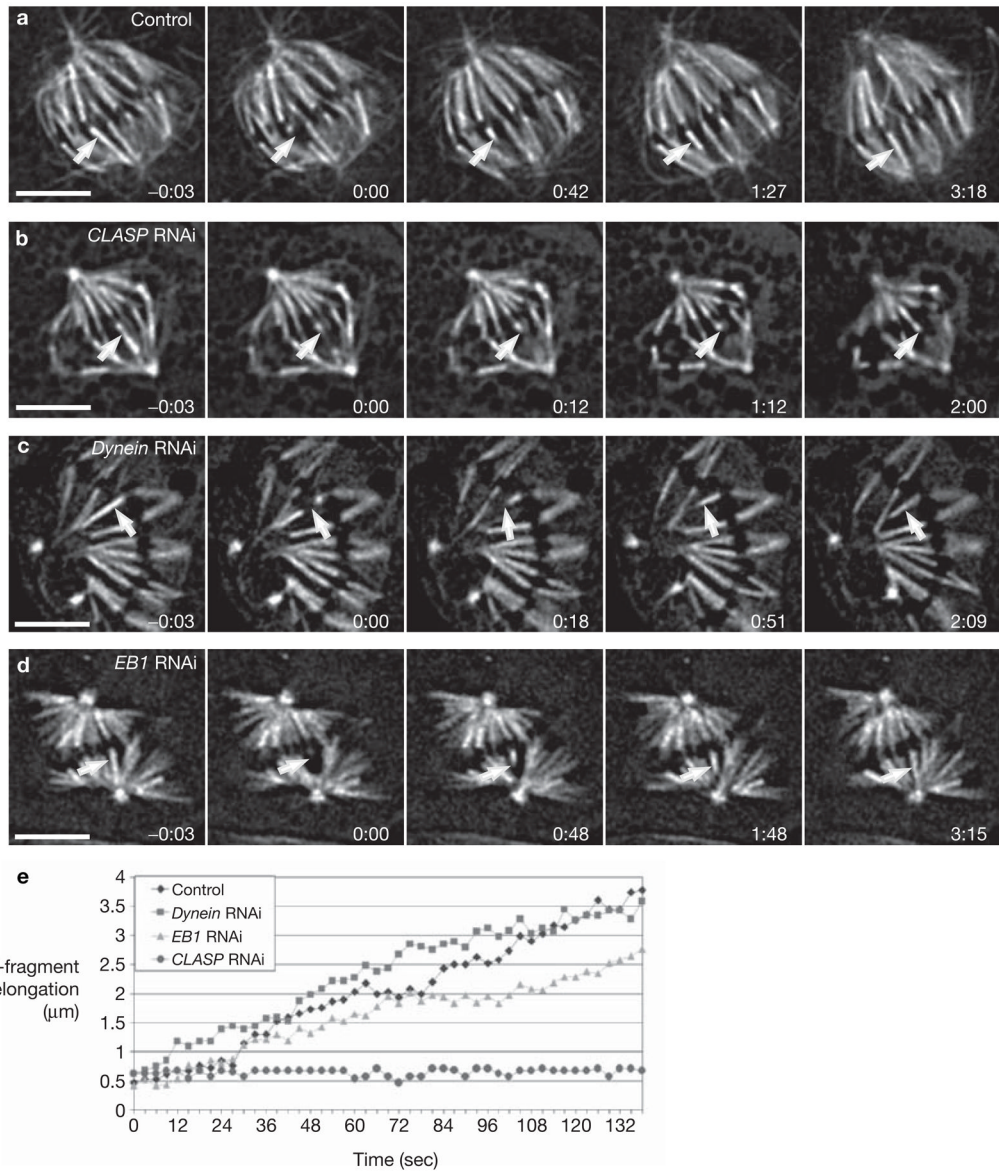


Figure 3. CLASP is required for subunit incorporation into K-fiber microtubules at their kinetochore-attached plus ends. **(a)** Control cell showing the re-growth (arrow) of a K-fiber after severing by laser microsurgery. **(b)** Similar to **a** but in a cell depleted of CLASP by RNAi. The severed K-fiber (arrow) does not re-grow even as the spindle gradually collapses. **(c, d)** K-fibers severed in dynein- or EB1-depleted cells, respectively, re-grow (arrow) at the same rates seen in control cells. Time is shown in min:sec; scale bar represents 5 μm. **(e)** K-fragment elongation curves after laser microsurgery for the cells shown in the previous panels. Movies of **a–d** can be found in the Supplementary Information.

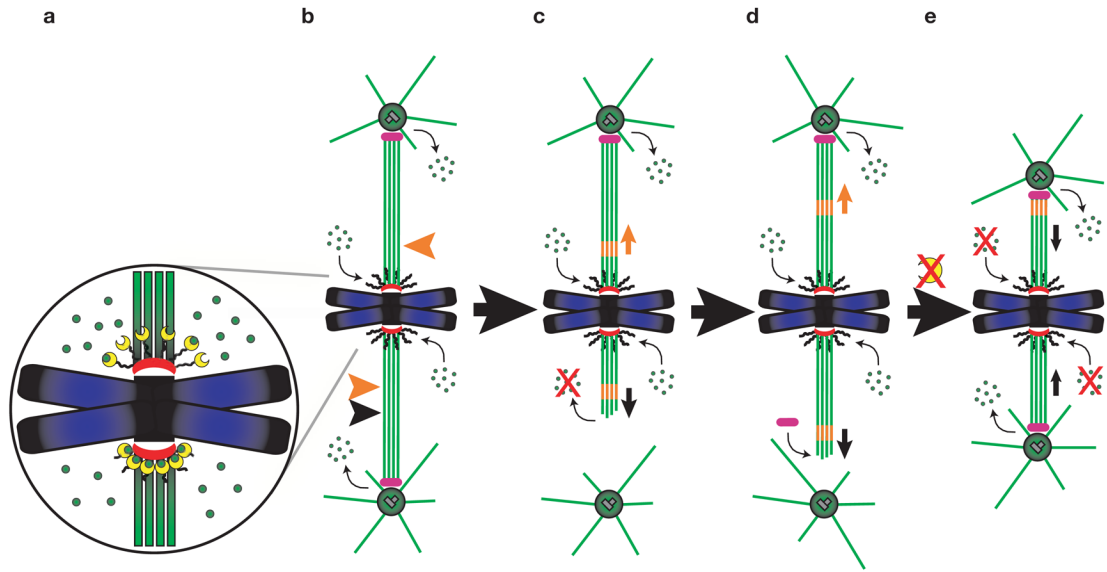


Figure 4. Schematic depicting the role of CLASP at kinetochores. (a) CLASP (yellow) mediates the incorporation of tubulin subunits (green) into kinetochore microtubule plus-ends at kinetochores. When a mature K-fibre is photobleached (top orange arrow **b–d**) the subunits move, or ‘flux’, poleward through the fibre and are removed near the poles. When a K-fibre is photobleached (bottom orange arrow in **b**) and then severed (black arrow) closer to the pole, the fibre re-grows towards the pole (**c–d**) by microtubule subunit incorporation at the kinetochore but not at the newly created microtubule minus ends, which are stable. Once the re-growing fibre matures, factors are recruited to its microtubule minus ends (**d**) (most probably Kinesin-13 family members such as KLP10A/Kif2a; pink) that allow for (and maybe power) subunit flux. In the absence of CLASP (**e**), the incorporation of subunits into mature K-fibre microtubule plus-ends is shut down, which inhibits flux. However, as microtubule minus-end depolymerization still occurs near the poles, the K-fibres shorten and the spindle collapses.

Rates of kinetochore-fragment elongation, pole-fragment shortening, flux, initial spindle collapse and anaphase chromosome motion

Table 1

Experiment	K-fragment elongation rate ($\mu\text{m min}^{-1}$)	P-fragment shortening rate ($\mu\text{m min}^{-1}$)	Flux rate ($\mu\text{m min}^{-1}$)	Initial spindle collapse rate ($\mu\text{m min}^{-1}$)	Anaphase poleward chromosome movement rate ($\mu\text{m min}^{-1}$)
Control	0.78 ± 0.17	21.80 ± 0.35	0.70 ± 0.15	—	1.24 ± 0.09
<i>CLASP</i> RNAi	~0	20.00 ± 4.81	~0	1.20 ± 0.06	—
<i>Dynein</i> RNAi	0.72 ± 0.12	19.93 ± 5.61	0.70 ± 0.18	—	—
EB1 RNAi	0.74 ± 0.12	19.93 ± 3.93	0.67 ± 0.12	—	—

Each rate represents the average \pm s.d. of five different events.