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Citation for published version:
Meireles, A, Dzhindzhev, N & Ohkura, H 2011, 'Kebab: Kinetochore and EB1 Associated Basic Protein That Dynamically Changes Its Localisation during Drosophila Mitosis' PLoS One, vol. 6, no. 9, e24174. DOI: 10.1371/journal.pone.0024174

Digital Object Identifier (DOI):
10.1371/journal.pone.0024174

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
PLoS One

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Kebab: Kinetochore and EB1 Associated Basic Protein That Dynamically Changes Its Localisation during Drosophila Mitosis

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Abstract

Microtubule plus ends are dynamic ends that interact with other cellular structures. Microtubule plus end tracking proteins are considered to play important roles in the regulation of microtubule plus ends. Recent studies revealed that EB1 is the central regulator for microtubule plus end tracking proteins by recruiting them to microtubule plus ends through direct interaction. Here we report the identification of a novel Drosophila protein, which we call Kebab (kinetochore and EB1 associated basic protein), through in vitro expression screening for EB1-interacting proteins. Kebab fused to GFP shows a novel pattern of dynamic localisation in mitosis. It localises to kinetochores weakly in metaphase and accumulates progressively during anaphase. In telophase, it associates with microtubules in central-spindle and centrosomal regions. The localisation to kinetochores depends on microtubules. The protein has a domain most similar to the atypical CH domain of Ndc80, and a coiled-coil domain. The interaction with EB1 is mediated by two SxIP motifs but is not required for localisation. Depletion of Kebab in cultured cells by RNA interference did not show obvious defects in mitotic progression or microtubule organisation. Generation of mutants lacking the kebab gene indicated that Kebab is dispensable for viability and fertility.

Introduction

The microtubule cytoskeleton is a dynamic network, constantly reorganising itself in response to various internal and external cues. In order to perform cellular functions as diverse as chromosome segregation, flagellar movement or neuronal transport, the microtubule network needs complex regulatory mechanisms [1]. Even though microtubule-associated proteins (MAPs) are regarded as the main regulators of microtubule organisation and dynamics [1], our knowledge of MAPs is still limited. Unlike microtubule motors, most non-motor MAPs do not have easily recognisable features within their primary sequence or high sequence conservation across eukaryotes. Furthermore, as hundreds of MAPs interact with microtubules even in a single cell, functional redundancies are likely to be very high. As the behaviour of microtubules varies within cells, and in different cell cycle stages and cell types, MAPs must be spatially and temporally regulated. Therefore we are still a long way from knowing the full complement of MAPs, how they regulate microtubules, and how they themselves are regulated in cells.

The microtubule is a polar filament made of tubulin dimers. The two ends, called plus and minus ends, behave differently from each other in vitro and in vivo [2]. The plus end is much more dynamic, and often interacts with other cellular structures, such as kinetochores and the cell cortex. Critically the interaction with other cellular structures influences the behaviour of the microtubule plus ends [3]. Therefore, microtubules can read the cellular environment to adopt an organisation specific to cell function. A specialised group of MAPs has been found to bind preferentially to microtubule plus ends, and are collectively called plus-end tracking proteins [3]. Plus-end tracking proteins are considered to be important for regulation of microtubule plus ends, and therefore have drawn much attention in recent years.

Recent studies highlighted the central role of EB1 among plus-end tracking proteins [4]. EB1 was originally identified as a binding partner of a tumour suppressor protein, adenomatous polyposis coli (APC), and later shown to track microtubule plus ends in cells [5], [6]. EB1 is a highly conserved protein across yeasts to humans [7], and is required for proper regulation of microtubule plus ends [4]. The central role for EB1 in microtubule plus end regulation has been demonstrated, as EB1 can track microtubule plus ends in the absence of other proteins in vitro, it physically interacts with many microtubule plus end tracking proteins, and is required for recruitment of these proteins to the microtubule plus ends in vivo [8], [9], [10], [11].

The conserved C-terminal domain of EB1 interacts with other microtubule plus end tracking proteins [12]. So far two motifs, the CAP-Gly domain and the linear motif SxIP, have been identified to interact with EB1 [13], [14]. However, interactions between EB1 and other microtubule plus end tracking proteins are very

Citation: Meireles AM, Dzhindzhev NS, Ohkura H (2011) Kebab: Kinetochore and EB1 Associated Basic Protein That Dynamically Changes Its Localisation during Drosophila Mitosis. PLoS ONE 6(9): e24174. doi:10.1371/journal.pone.0024174

Editor: Debra L. Silver, Duke University Medical Center, United States of America

Received June 8, 2011; Accepted August 1, 2011; Published September 2, 2011

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Funding: This study is funded by The Wellcome Trust (081849; www.wellcome.ac.uk). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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The conserved C-terminal domain of EB1 interacts with other microtubule plus end tracking proteins [12]. So far two motifs, the CAP-Gly domain and the linear motif SxIP, have been identified to interact with EB1 [13], [14]. However, interactions between EB1 and other microtubule plus end tracking proteins are very
dynamic, and are usually undetectable by co-immunoprecipitation from cell extracts [15]. Nevertheless, proteins interacting with EB1 have been successfully identified by mass-spectrometry after pull-down from cell extract using bacterially produced EB1 proteins [16], [17], [18], [19], [20]. These EB1 interacting proteins have been shown to play important roles in various aspects of microtubule regulation [17]. Further identification of EB1 interacting proteins will be crucial for a full understanding of microtubule regulation.

Here we report the identification of a novel EB1 interacting protein, which we call Kebab, from Drosophila melanogaster, by an in vitro expression screen. We found that Kebab shows a unique dynamic localisation during mitotic progression. It localises to kinetochores during mitosis, where it progressively accumulates during anaphase. In telophase it associates with microtubules. Kebab interacts with EB1 through two SxIP motifs, but this interaction is not required for Kebab localisation. Kebab is dispensable for viability and fertility in flies.

**Results**

**Identification of a novel EB1-interacting protein, Kebab, in Drosophila**

To gain further insight into the regulation of microtubule plus ends, we have identified a novel EB1-interacting protein, CG31672, that associates with mitotic kinetochores. The gene is located at 22C1 on chromosome arm 2L, and the predicted molecular weight of the protein is 64 kDa. As it is a very basic (pI = 9.7), we called this previously uncharacterised protein Kebab (abbreviated as to Keb), which stands for kinetochore and EB1 associated basic protein.

We identified this EB1-interacting protein through in vitro expression cloning using a collection of unique annotated Drosophila cDNAs (DIVEC; [21]). In brief, cDNAs from the collection were transcribed and translated in vitro. Bacterially produced MBP (maltose binding protein) alone or MBP-EB1 was incubated with the translated product, and pulled down to assay specific interaction with EB1. These putative EB1-interacting proteins were further examined for their subcellular localisation in a Drosophila cultured S2 cell line originated from embryos. Because of the unique localisation, Kebab was selected for further study. Interaction between Kebab protein and EB1 was further confirmed by pull down of Kebab from S2 cell extract by MBP-EB1 (Figure 1).

**Kebab associates with kinetochores and spindle microtubules**

To analyse its subcellular localisation, Kebab was fused to GFP and expressed in a Drosophila cultured S2 cell line. Kebab protein fused to GFP showed dynamic localisation to the mitotic apparatus during mitosis. The localisation is identical using both N and C-terminal GFP fusions, suggesting it reflects the native localisation of Kebab protein. During mitosis, it is localised to multiple foci on chromosomes, which possibly correspond to kinetochores (Figure 2). To confirm this possibility, Kebab fused with GFP was co-stained with the centromere protein Cid (the Drosophila CenpA homologue). GFP foci were found to overlap with Cid foci (Figure 2A). Closer inspection showed that Kebab-GFP foci were located slightly outside each pair of Cid foci in metaphase, indicating Kebab is associated to kinetochores (Figure 2B). Kebab stayed on kinetochores during anaphase, and the signals appeared to intensify in late anaphase (Figure 2C). From late anaphase to telophase, it was also associated with residual spindle microtubules between the separated chromosomes (Figure 2D). In interphase, it

**Dynamic localisation of Kebab during mitosis**

To follow the changing localisation of Kebab during mitosis in live cells, a stable cell line simultaneously expressing Kebab-GFP and mCherry-α-tubulin was generated and observed under a spinning disc confocal microscope (Movie S1; Figure 3A-C). In metaphase, Kebab localised to kinetochores, although the signal was weak (1 in Figure 3A). During anaphase, it progressively accumulated on kinetochores (2 in Figure 3A,B). In late anaphase, it also started to localise to microtubules notably in the central spindle and centrosomal regions (3 in Figure 3A,B). The intensity increased further when the cell entered telophase (4, 5 in Figure 3A,B).

To further analyse the accumulation of Kebab on kinetochores during anaphase, the intensity of Kebab-GFP on kinetochores was quantified over time. Small equal-sized circles were drawn around kinetochores and in the cytoplasm, and the total pixel intensity within each circle was measured. The mean values of kinetochore signals above the cytoplasmic signal were plotted against time after normalisation. Although patterns of accumulation are not

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**Figure 1. Kebab interacts with EB1.** Cell extract from a stable cell line expressing Kebab-GFP was incubated with bacterially produced MBP and MBP-EB1. MBP and MBP-EB1 were pulled down and subjected to western blot using an anti-Kebab antibody (the upper panel). One twentieth of the cell extract was run, relative to the pull-down fractions. Protein staining of the same membrane is shown in the lower panel. Kebab-GFP is specifically pulled down with EB1. doi:10.1371/journal.pone.0024174.g001

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identical, the signals gradually increased throughout anaphase in all cells examined (Figure 3D).

Kebab has an atypical CH domain, a coiled-coil region and EB1 binding motifs

To gain further insight into the Kebab protein, we investigated the existence of structural domains in Kebab using bioinformatic tools (Figure 4A). Clear orthologues which share a significant homology to Kebab over the entire region were found in the genomes of *Drosophila* species, but not beyond. The middle region (amino acids 238–373) was found to have a weak but significant similarity to the calponin homology (CH) domain of the human kinetochore protein Ndc80 and its orthologues (Figure 4B), but not to CH domains of other proteins. The CH domains of Ndc80 are well diverged from other CH domains at the primary sequence level. The homology was only recognised after determination of crystal structures, and is considered to contribute to microtubule binding [22], [23]. The residues of the human Ndc80 CH domain involved in microtubule binding (asterisks in Figure 3B; [23]) are often conserved in Kebab. Therefore this domain of Kebab and the CH domain of Ndc80 appear to form a subfamily of CH domains.

In addition to this CH domain, extensive coiled coils were predicted in the C-terminal residues (385–411 aa; 436–456 aa; 513–539 aa). Kebab has two regions near the CH domain (at 150, 169) which match an SxIP motif (S/TxIP) which is a known EB1 binding motif (Figure 4A). The other known EB1 binding motif, CAP-Gly domain [13], was not found.

Kinetochore and microtubule localisation is independent of EB1 binding

To define the regions responsible for the localisation of Kebab, a series of truncated proteins with a GFP tag were expressed in S2 cells and examined for their localisation by immunostaining using an anti-GFP antibody (Figure 4C, D; Figure S2). A series of truncations at the N-terminus suggested that the region between...
the 101st and 254th residues is important for both kinetochore and microtubule localisation. A series of truncations at the C-terminus revealed that the N-terminal 254 residues are sufficient for minimal localisation to kinetochores and microtubules. Kebab lacking most (268–370) of the CH domain was still able to bind to kinetochores and microtubules, indicating that a full CH domain is not essential for localisation.

It is possible that the two potential EB1 binding motifs (SxIP) located close to the CH domain may be involved in microtubule or kinetochore localisation. To experimentally test whether these SxIP motifs are responsible for EB1 binding of Kebab, a mutation was introduced to one or both of the SxIP motifs in a full-length protein. The mutants, together with the wild-type protein, were translated in vitro and tested for binding to MBP-EB1 and MBP alone by pull down assay (Figure 4D). A mutation in the first motif greatly reduced the binding to EB1, while a mutation in the second one slightly reduced it. Double mutants further reduced EB1 binding to the minimum level. These results showed that the two SxIP motifs function additively in the interaction with EB1 and the motifs together contribute to most, if not all, of Kebab’s interaction with EB1.

Next, to test the contribution of EB1 interaction for the localisation, a full-length Kebab with a mutation in SxIP motifs was expressed in S2 cells (Figure 4C,E). Kinetochore localisation of Kebab was unaffected by either single mutations or the double mutation. Furthermore, EB1 depletion by RNAi did not change kinetochore localisation of Kebab (Figure 4F). This showed that Kebab kinetochore localisation does not require EB1 interaction.

Kebab is dispensable for mitotic progression

To understand its cellular role, Kebab was depleted from S2 cells by RNA interference (RNAi). RNAi using double-stranded RNA (dsRNA) is known to work robustly in S2 cells [24], [25].
The effectiveness of RNAi was confirmed by western blots on a cell line expressing Kebab-GFP treated with the dsRNAs (Figure S3). Three dsRNAs, each corresponding to non-overlapping regions of the kebab transcript were used and gave essentially identical results. These cells were immunostained for a-tubulin, mitotic specific phospho-H3 (at serine 10) and DNA. The mitotic index was calculated as the frequency of phospho-H3 positive cells. Each mitotic figure was categorised and counted. These studies did not reveal a significant difference between kebab and control RNAi (Figure 5A).

To test the involvement of Kebab in the spindle checkpoint, cells depleted of Kebab were challenged by incubation with colchicine, a microtubule depolymerising drug. In control cells, the mitotic index increased after incubation with colchicine. In Kebab depleted cells, the mitotic index also increased to a level similar to the control after colchicine incubation (Figure 5A).

To detect more subtle defects in the timing of mitotic progression, live imaging was carried out using cells stably expressing GFP-a-tubulin. The cells were treated with dsRNA for kebab or a control. The time was measured from nuclear envelope breakdown to the onset of anaphase (Figure 5B). There was no significant difference between kebab RNAi and the control. Although no firm conclusions can be drawn from RNAi experiments, these studies suggest that Kebab is dispensable for mitotic progression in S2 cells.

Flies lacking Kebab are viable and fertile

To understand the role of Kebab in developing flies, deletion mutants of Kebab were generated. We took advantage of a line in which a P-element is inserted into the 5’ non-coding region of the kebab gene. The P-element was remobilised and the presence/absence of the genomic regions surrounding the P-element was confirmed by western blots on a cell line expressing Kebab-GFP treated with the dsRNAs (Figure S3). Three dsRNAs, each corresponding to non-overlapping regions of the kebab transcript were used and gave essentially identical results. These cells were immunostained for a-tubulin, mitotic specific phospho-H3 (at serine 10) and DNA. The mitotic index was calculated as the frequency of phospho-H3 positive cells. Each mitotic figure was categorised and counted. These studies did not reveal a significant difference between kebab and control RNAi (Figure 5A).

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progeny without the Y chromosome, or females with the Y chromosome, aneuploidy in gametes would give rise to viable male by the number of X chromosomes independently of the Y frequency of aneuploidy in the viable progeny was not significantly keb deletion mutant males were crossed with wild type, the chromosomes in germ lines. As the sex in accurately in the mutant, we examined segregation of the sex fertility. results demonstrated that Kebab is not essential for viability and fertility. Furthermore, a homozygous stock mutants, including one completely lacking the coding region were identified. These kebab coding region is completely deleted without affecting microtubules becomes more prominent in telophase. This microtubules or centrosomal regions. The association with progression. In late anaphase, Kebab starts localising to spindle kinetochores along microtubules. In either case, it is a very interesting and unusual property of a kinetochore protein. EB1 polymerisation state. Alternatively, Kebab is transported to kinetochore-associated microtubule plus ends regardless of the one of the sister kinetochores moving away from poles, which is localisation to outer kinetochores. The most notable feature of Kebab is that it progressively accumulates on kinetochores during anaphase. Although this behaviour is unusual among kinetochore proteins, a group of centromere proteins, including CenpA, CenpC and Mis18, have been reported to progressively accumulate on centromeres during anaphase [29], [30], [31]. These proteins interact with each other to define the centromere identity through loading of CenpA, a Histone 3 variant, on DNA [29]. It is still not understood why this occurs during anaphase. Interestingly, Kebab is not colocalised with CenpA, rather is localised outside of CenpA. Although all kinetochore proteins require CenpA for their localisation, generally they do not increase in intensity during anaphase. It is possible that Kebab recruitment to kinetochores may be somehow linked to CenpA loading, or the dose of CenpA on centromeres, rather than other kinetochore proteins. Alternatively, Kebab may be regulated by a previously unknown mechanism. The second interesting property of Kebab is that microtubules are required for its kinetochore localisation. This property is unusual but not unique among kinetochore proteins. EB1 localisation to kinetochores in PtK1 cells was previously reported to depend on microtubules [32]. It was found to localise to only one of the sister kinetochores moving away from poles, which is coupled to microtubule polymerisation. In contrast, Kebab localisation appears to be symmetrical in metaphase and become more prominent when microtubules are depolymerising in anaphase. Other kinetochore proteins such as Ska1 and Ajuba are also reported to depend on microtubules for their localisation [33], [34]. The mechanism and significance of this microtubule dependency are still under speculation. Kebab may recognise kinetochore-associated microtubule plus ends regardless of the polymerisation state. Alternatively, Kebab is transported to kinetochores along microtubules. In either case, it is a very interesting and unusual property of a kinetochore protein. The localisation of Kebab dynamically changes during mitotic progression. In late anaphase, Kebab starts localising to spindle microtubules or centromosomal regions. The association with microtubules becomes more prominent in telophase. This changing pattern of localisation is unique among previously reported kinetochore proteins or microtubule-associated proteins.

**Discussion**

EB1 regulates microtubule plus ends through interaction with multiple proteins [26]. In this study we identified a novel EB1-interacting protein, Kebab, which shows a dynamic localisation to kinetochores and microtubules in mitosis. Previously several studies have successfully identified EB1-interacting proteins by mass-spectrometry after pull down using bacterially produced EB1 protein [20]. One drawback of this approach is that the chance of a protein being identified depends on its level in a particular tissue or cell line. We have identified Kebab by *in vitro* expression cloning. *In vitro* expression cloning was originally developed by Lustig and co-workers, using random cDNAs from *Xenopus* eggs [27], [28]. It was later adapted for use with a collection of annotated unique *Drosophila* cDNAs [21]. This study looked for substrates of a kinase by examining the shift in gel mobility after kinase reaction. We further adapted this method for a pull down assay to identify interacting proteins. The advantage of this approach over a mass-spectrometry based one would be that low abundance proteins or those expressed in specific cell types have equal chance of being identified, as long as cDNA has been isolated from some tissues.

We found that Kebab localises to kinetochores during mitosis. The relative position of Kebab to the *Drosophila* CenpA orthologue Cid, suggests it localises to outer kinetochores. The most notable feature of Kebab is that it progressively accumulates on kinetochores during anaphase. Although this behaviour is unusual among kinetochore proteins, a group of centromere proteins, including CenpA, CenpC and Mis18, have been reported to progressively accumulate on centromeres during anaphase [29], [30], [31]. These proteins interact with each other to define the centromere identity through loading of CenpA, a Histone 3 variant, on DNA [29]. It is still not understood why this occurs during anaphase. Interestingly, Kebab is not colocalised with CenpA, rather is localised outside of CenpA. Although all kinetochore proteins require CenpA for their localisation, generally they do not increase in intensity during anaphase. It is possible that Kebab recruitment to kinetochores may be somehow linked to CenpA loading, or the dose of CenpA on centromeres, rather than other kinetochore proteins. Alternatively, Kebab may be regulated by a previously unknown mechanism.

To genetically test whether chromosomes are segregated accurately in the mutant, we examined segregation of the sex chromosomes in germ lines. As the sex in *Drosophila* is determined by the number of X chromosomes independently of the Y chromosome, aneuploidy in gametes would give rise to viable male progeny without the Y chromosome, or females with the Y chromosome. A B*-marked Y chromosome was used to monitor the inheritance of the Y chromosome.

When keb mutant females were crossed with wild type, aneuploidy in the viable progeny appeared more frequent (1.19±2.20% in keb vs 0.17±0.53% in wild type), but the difference is not statistically significant (p = 0.07). Also, when the keb deletion mutant males were crossed with wild type, the frequency of aneuploidy in the viable progeny was not significantly different from a control (0.18±0.68% in keb vs 0.89±1.22% in wild type; p = 0.07).

In conclusion, the removal of Kebab has little detrimental effect on a cultured cell line or in developing flies.

Figure 5. Kebab is dispensable for mitotic progression and fly viability and fertility. (A) Mitotic index of S2 cells without or after colchicine incubation. S2 cells were subjected to RNAi of kebab and a control. S2 cells were immunostained for phospho-H3 at Serine 10 and DNA. The mitotic index was the proportion of phospho-H3 positive cells. Bars indicate standard deviations. (B) Time from nuclear envelope breakdown to anaphase onset. S2 cells expressing mCherry-RCC1 were subjected to RNAi of kebab and a control, and observed in live cells. (C) The genomic region around kebab. The open boxes, grey boxes and kinked lines indicate non-coding exons, coding exons and introns, respectively. The triangle indicates the position of the P-element insertion in k09932. The parentheses indicate regions deleted in each keb mutant. doi:10.1371/journal.pone.0024174.g005

examined by PCR. We have isolated a mutant (Δ22) in which the kebab coding region is completely deleted without affecting neighbouring genes (Figure 3C; Figure S4). In addition, multiple lines lacking most of the kebab coding region were identified. These mutants, including one completely lacking the kebab gene, were viable and fertile in both sexes. Furthermore, a homozygous stock can be maintained without difficulty for many generations. These results demonstrated that Kebab is not essential for viability and fertility.

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Nevertheless, other types of proteins are known to change localisation during mitotic progression. For example, the chromosomal passenger complex localises to centromeres/kinetochores until metaphase and relocates to microtubules in the spindle midzone at the onset of anaphase [35]. This change in localisation is considered to be crucial for the change in kinetochore behaviour at the onset of anaphase, and stabilisation of the spindle midzone in telophase [35]. Dynamic localisation of Kebab may subtly influence a change in behaviour of kinetochore or spindle microtubules.

We showed that Kebab can directly bind to EB1 in vitro. Interaction with EB1 is mediated by two SxIP motifs located near the CH domain. The SxIP motif is a linear motif found in many EB1 binding proteins [14]. Mutations in both SxIP did not abolish the localisation of Kebab, suggesting interaction with EB1 is not essential for localisation. Consistently we found that EB1 depletion did not disrupt the localisation of Kebab. It is possible that EB1-independent localisation masks the EB1 dependent localisation in a specific location. Alternatively EB1 interaction may be important for Kebab function rather than the localisation. Further studies are needed to clarify the significance of EB1 interaction.

Kebab contains a domain which is most similar to the atypical CH domain of another kinetochore protein, Ndc80. Ndc80 is one of the critical proteins which connect kinetochores to microtubules [36]. This domain of Ndc80 is considered to be the microtubule binding domain [23]. The CH domain of Ndc80 is quite distinct from typical CH domains found in other proteins, and was only recognised after the crystal structure was determined [22]. Our observations of Kebab have been made in regimens that either first introduced the S. melanimosger genome, there may be proteins that function redundantly with Kebab. Hundreds of proteins in each cell type can bind to microtubules, and collectively determine their behaviour. These form a diverse array of proteins, with only a small minority containing known microtubule binding motifs. It is likely that many structurally distinct proteins can function redundantly to regulate microtubules. For example, microtubule bundling can be achieved by many microtubule or protein complexes which contain multiple microtubule binding sites. It is a challenge in biology to understand a system that involves many redundancies, such as microtubule regulation. Future identification of proteins that have overlapping function with Kebab will shed light on the function and regulation of Kebab protein in mitosis.

**Materials and Methods**

**Molecular and protein techniques**

Standard DNA manipulation and protein techniques were used [37]. The kebab coding region was introduced first into the Gateway entry vector pDONR221, and then into destination vectors, pAGW and pAWG to generate a plasmid for expression of Kebab fused to GFP either N or C-term under the actin5C promoter. Kebab deletions were created by PCR amplification of gateway expression clone with primers flanking the regions to be deleted and carrying an EcoRI site. Digestion with EcoRI and ligation generated the desired plasmid which was subsequently sequenced. Premature stop codons were introduced by site directed mutagenesis using Quick Change XL site directed mutagenesis kit (Agilent), following manufacturer’s instructions.

**Identification of Kebab**

Kebab was identified by *Drosophila in vitro* expression cloning as described below. A pool of 12 cDNAs from a *Drosophila Gold Gene Collection* was transcribed and translated in *Drosophila* using the T7 TriT Quick Coupled system (Promega) in the presence of 35S-S-methionine (Easytag, Perkin Elmer). Each translated product was split into two and incubated in DIVEC buffer (50 mM Hepes pH 7.6, 1 mM MgCl2, 1 mM EGTA, 200 mM NaCl, 0.5% TritonX100) for 60 minutes with amylase resin (New England Biolabs) coupled with bacterially-produced MBP or MBP-EB1. After extensive washing in DIVEC buffer, the beads were boiled with the sample buffer and run on an SDS gel. Dried gels were exposed to X-ray film (Hyperfilm, GE Healthcare). cDNA pools which gave bands specific for MBP-EB1 pull down were further studied by testing sub-pools until a single responsible cDNA was identified.

**Cell culture**

*Drosophila* Schneider S2 cells were cultured and RNA interference (RNAi) was performed according to published methods [25]. Plasmids were transfected using Effectene transfection reagent (Qiagen) following manual’s instructions. A cell line stably expressing GFP was a kind gift from Ron Vale [38]. Cell lines expressing GFP-Kebab and/or mCherry-tubulin were established by basticidin selection (25 μg/ml) after co-transfection with resistance vector (pCoBlast, Invitrogen). Double-stranded RNA (dsRNA) corresponding to regions amplified by primer pairs (forward/reverse) 5'-GTAGTATGCTAATGCG-3'/5'-CTC-TTTTGAAGTTCTTCG-3', 5'-ACAATTACAAATCTC-3'/5'-CTAAGGGCTTCCCTGGG-3', and 5'-GGGAAGAT-AAGGACAC-3'/5'-CAATTATGCTAACC-3' of kebab were used. dsRNA corresponding to *E. coli* β-lactamase was used as a control.

**EB1 pull-down assay**

MBP and MBP-EB1 were bacterially produced, purified and bound to amylace resin (New England Biolabs). About 1×10^7 S2 cells expressing Kebab-GFP were resuspended in DIVEC buffer (50 mM Hepes pH 7.6, 1 mM MgCl2, 1 mM EGTA, 200 mM NaCl, 0.5% TritonX100) and sonicated for 1 min in pulses of 1 s ON 5 s OFF. The extract was incubated with the beads for 1 hr at 4°C with rotation. After extensive washing in DIVEC buffer, the beads were boiled with the sample buffer and run on an SDS gel.

**Cytological analysis**

Immunostaining of S2 cells was carried out and examined as previously described [39]. Briefly, S2 cells were plated on ConcanaavalinA (ConA) coated coverslips and after 2 hrs were fixed with 90% methanol, 3% formaldehyde, 5 mM NaHCO3 pH 9 at −80°C. Cells treated with colchicine (2 μM, Sigma) and colcemid (5 μM, Sigma) were fixed after 2 hrs treatment. Cells treated with Paclitaxel (1 μM, ICN) were fixed after 10 or 30 min treatments. Antibodies against mouse α-tubulin (DM1A, 1:200, Sigma), rabbit α-GFP (1:500, Molecular Probes), mouse α-GFP (3E6, 1:500, Molecular Probes), mouse α-Cd (1:100, AbCam) and rabbit α -histone-H3-phosphate (1:500, Upstate) were used as primary antibodies. Images were captured using a Zeiss Axioplan 2 microscope equipped with a CCD camera (Hamamatsu) controlled by Openlab software (Perkin Elmer).

**Live imaging**

S2 cells were plated in ConA coated MatTek glass-bottom dishes, in culture media, for 2 hrs. Samples were examined at
room temperature by a microscope (Axiovert; Carl Zeiss) attached to a spinning-disc confocal head (Yokogawa) using Volocity (PerkinElmer). Images were acquired once every 30 s (analysis of mitotic progression) or once every 60 s (analysis of kebab accumulation at kinetochores). For quantification of Kebab at kinetochores, 4 circles of 0.7 μm² were drawn and used to manually track the brightest kinetochores and to establish background values. After background subtraction, mean intensities were normalised against the highest value for each sample, and plotted against time. Time zero was set to anaphase onset. A kymograph was generated by making maximum intensity projections first onto the X-Y plane and then onto the long axis of the spindle for each time point, and aligning this one dimension data against time as the second dimension.

**Fly techniques**

Standard fly techniques were used [40]. w^{1188} was used as wild type in this study. keb mutants were generated by remobilisation of a P-element (k09932) inserted near the coding region. The transposase gene D2–3 was introduced into P[icerW]k09932 by crossing. Chromosomes which have lost the w' gene on the P-element were selected and tested over a deficiency uncovering the keb gene. No chromosomes lethal over the deficiency were isolated. Viable chromosomes were tested over the deficiency for the presence of the keb genomic region by PCR. Once the stocks were established, the breakpoints were determined by further PCR.

Frequencies of sex chromosome aneuploidy were genetically determined using B'– marked Y chromosomes. In brief, 21–42 wild-type or mutant females were individually crossed with otherwise wild-type males carrying a B'-marked Y chromosome and the progeny were counted for aneuploidy (B females or non-B males). In reciprocal crosses, wild-type females were individually crossed with wild-type or mutant males carrying the B'-marked Y chromosome. Frequencies of aneuploidy from individual crosses were calculated, and the averages and standard deviations were determined for wild-type and the mutant. The Wilcoxon test was used to estimate the statistical significance of differences between the mutant and wild type.

**Supporting Information**

**Movie S1**  S2 cells expressing Kebab-GFP and mCherry-α-tubulin. See Figure 3 for details. (MOV)

**Figure S1**  Kebab localises to the cytoplasm in interphase. S2 cells were transfected with a plasmid expressing Kebab-GFP under the actin promoter, and immunostained for GFP, α-tubulin and DNA. Bar = 10 μm.

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**Figure S2** Various mutations and truncations affect Kebab localisation. A series of truncations and mutations were tested for Kebab localisation, as outlined for Figure 4. A representative image for each construct is shown to highlight the presence or absence of kinetochore localisation.

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**Figure S3**  RNAi of kebab is effective. S2 cells stably expressing Kebab-GFP were treated with the dsRNAs used in this study. A western blot was carried out using an anti-GFP antibody (the upper panel) and the same membrane was stained for protein (the lower panel). Expression construct for Kebab-GFP does not contain the endogenous kebab 3’UTR and therefore was resistant to RNAi using dsRNA (#5) corresponding to the kebab 3’UTR. The other dsRNAs (#2, #4) correspond to the kebab coding region, and effectively depleted Kebab-GFP.

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**Figure S4**  The kebab gene is deleted from Δ22. (A) A diagram showing the genomic region around the kebab gene. Thick bars indicate the regions which PCR primer pairs would amplify. (B) PCR was carried out to define the genomic region absent in a putative deletion line (Δ22) generated by remobilisation of the P-element k09932. Genomic DNA was prepared from a male fly with Δ22 over the deficiency D(2L)ED125 lacking the entire region surrounding the kebab gene, together with a wild-type control (w) and other putative deletions. PCR was carried out using each primer pair shown in A. The regions b and c are missing from Δ22, but a and d are intact.

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**Acknowledgments**

The authors thank Agata Gluszek and Laura Coulter for assisting with experiments, Sally Beard for helpful comments on the manuscript, Roger Tsien and Ronald Vale for kind gifts of the mCherry clone and a GFP-tubulin cell line, and Bloomington Drosophila Stock Center and Drosophila Genome Resource Center for fly stocks and clones.

**Author Contributions**

Conceived and designed the experiments: AMM NSD HO. Performed the experiments: AMM NSD. Analyzed the data: AMM NSD HO. Contributed reagents/materials/analysis tools: AMM NSD HO. Wrote the paper: AMM HO.

**References**


