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The Human Kinesin-14 HSET Tracks the Tips of Growing Microtubules in Vitro

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Tip-tracking of kinesin-14 motor proteins is believed to be crucial for the assembly and maintenance of dynamic microtubule arrays. However, in contrast to other members of the kinesin-14 family, *H. sapiens* kinesin-14 HSET has so far never been observed to be prominently located at microtubule plus ends. Here, using an in vitro microtubule dynamics reconstitution assay we observe tip-tracking of GFP-HSET in the presence of *H. sapiens* EB1 (hsEB1). Tip-tracking depended on the SxIP-like motif in HSET as well as on the EB homology domain in hsEB1. *D. melanogaster* Ncd and *S. pombe* Klp2 tip-tracking reconstitution assays accompanied by kinesin-14 amino acid sequence comparisons suggest that SxIP-like motif mediated tip-tracking dependent on EB family proteins is conserved in the kinesin-14 family of molecular motors. © 2013 Wiley Periodicals, Inc.

Key Words: HSET; EB1; kinesin-14; microtubule; tip-tracking

Introduction

Members of the kinesin-14 family are homodimeric motor proteins, which generate ATP-dependent, nonprocessive movement toward the minus-ends of microtubules. The human kinesin-14 HSET in vivo is localized predominantly to microtubule lattices where it cross-links microtubules and regulates spindle length during mitosis by sliding microtubules relative to each other [Cai et al., 2009]. In contrast to other members of the kinesin-14 family, such as *S. pombe* kinesin-14 Klp2, *D. melanogaster* kinesin-14 Ncd, and *A. thaliana* ATK5 [Ambrose et al., 2005; Goshima et al., 2005; Janson et al., 2007], HSET has not been observed to track the tips of growing microtubules [Cai et al., 2009]. This is surprising because kinesin-14 tip-tracking on the ends of dynamic microtubules is believed to be crucial for a number of intracellular functions. For example, tip-tracking of Klp2 and Ncd is proposed to be involved in the stabilization of *S. pombe* interphase-microtubule-arrays and the focusing of kinetochore-fibers at spindle poles in *D. melanogaster*, respectively [Goshima et al., 2005; Janson et al., 2007]. In vivo, it has been shown that Ncd tip-tracking is mediated by *D. melanogaster* EB1 [Goshima et al., 2005], a member of the EB protein family known to target a variety of proteins to microtubule tips [Akhmanova and Steinmetz, 2008; Kumar and Wittmann, 2012]. One way of interacting with EB1 is provided by short SxIP-like polypeptide motifs present in the amino acid sequence of many EB1-binding proteins [Fong et al., 2009; Honnappa et al., 2009; Buey et al., 2012; Jiang et al., 2012). It is not known, however, if EB1 dependent tip-tracking, mediated by the SxIP-like motif, is a general mechanism of kinesin-14 interaction with microtubules. Here we investigate this issue using a dynamic-microtubule reconstitution assay based on dual-color total-internal reflection fluorescence (TIRF) microscopy.

Material and Methods

In Vitro Microtubule Dynamics Assay

Microtubule seeds were polymerized in the presence of 0.5 mM GMP-CPP (Jena Bioscience) at 37°C for 30 min using a mixture of biotinylated, rhodamine labeled and unlabeled pig brain tubulin (1:2:47, final concentration 4 mg/ml). Flow chambers with hydrophobic glass surfaces were prepared as described previously [Fink et al., 2009]. Antibiotin antibodies (Sigma) 1% in PBS were incubated

Additional Supporting Information may be found in the online version of this article.
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for 5–10 min followed by 15 min incubation of 1% Pluronic F127 (Sigma) in PBS. Biotinylated microtubule seeds in BRB80 (80 mM Pipes/KOH pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA) were allowed to bind to the surface-attached antibodies for 5 min. Channels were rinsed once with BRB80 and then once with assay buffer (see below). In the next step, GFP-HSET, EB1 proteins and 22 μM pig brain tubulin (16% fluorescently labeled with rhodamine) in assay buffer (20 mM HEPES at pH 7.2, 150 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, 1 mM ATP or 1 mM ADP, 10 mM dithiothreitol, 0.5 mg ml^{-1} casein, 0.1% Tween, 0.1% w/v methylcellulose, 20 mM D-glucose, 220 μg ml^{-1} glucose oxidase and 20 μg ml^{-1} catalase) were flushed into the flow cell at final assay concentrations as indicated in the figure legends and imaging was started. All experiments were performed at 24°C.

Imaging

Rhodamine-labeled microtubules and GFP-labeled HSET were visualized sequentially by switching between TRITC (tetramethyl rhodamine isothiocyanate) and GFP channels: solid-state laser 532 nm (Cobolt), TRITC filter cube (Chroma Technology) and solid-state laser 488 nm (Vortran Stradus), GFP filter cube (Chroma Technology). Images were acquired by the MetaMorph software package (Universal Imaging) using a EMCCD camera (Ixon DV 897, Andor) mounted on a inverted fluorescence microscope (Axiovert 200M, Zeiss) equipped with an Alpha Plan-Apochromat 64× oil 1.46 NA DIC objective (Zeiss), a TIRF-slider (Zeiss) and an autofocus (Zeiss) at an acquisition rate of 1 frame per 3 sec.

Protein Expression and Purification

N-terminal hexa-histidine-tagged GFP-HSET and GFP-HSET-SQNN (generated using the Quickchange Lightning Kit, Quiagen) were expressed in Drosophila SF9 insect cells using the Bac-to-Bac Expression System (Invitrogen). Harvested cells were resuspended in buffer A (50 mM sodium phosphate buffer pH 7.5, 1 mM MgCl2, 10 mM 2-mercaptoethanol, 300 mM NaCl, 0.1% Tween20 w/vol, 10% glycerol w/vol, 30 mM imidazole and EDTA-free protease inhibitors (Roche). Crude lysate was centrifuged at 20,000*g* at 4°C and loaded on NiNTA resin (Qiagen). The resin was washed with buffer A containing 60 mM imidazole. Proteins were eluted in buffer A containing 300 mM imidazole. The recombinant C-terminal hexa-histidine tagged fusion proteins hsEB1, dmEB1, Mal3, GFP-Ncd, GFP-Ncd-1–349, GFP-Klp2 and GFP-Klp2-SHNN-SNNN (generated using the Quickchange Lightning Kit, Quiagen) were expressed in *E. coli* BL21-CodonPlus®(DE3)-RIPL (Stratagene) induced with 0.5 mM IPTG for 16 h at 15°C. Harvested cells were resuspended in buffer A, lysed using an EmulsiFlex high pressure homogenizer (Avestin) at 4°C, and hexa-histidine-tag purified as described above. All proteins were snap-frozen in liquid nitrogen and stored at −80°C.

Results and Discussion

To reconstitute kinesin-14 interaction with dynamic microtubules *in vitro* we initiated microtubule growth from fluorescently labeled microtubule seeds immobilized on passivated surfaces. GFP-labeled HSET, unlabeled EB1, and rhodamine-labeled tubulin in presence of 1 mM ATP and 1 mM GTP (see Methods for details) were flushed into the flow-chamber. HSET localization and microtubule dynamics were visualized by TIRF microscopy (Fig. 1A). *H. sapiens* EB1 (hsEB1) has previously been described to autonomously track the tips of growing microtubules in vitro [Bieling et al., 2008]. We here found that GFP-HSET tip-tracks in presence of unlabeled hsEB1 (Fig. 1B). ATPase activity of the motor was not necessary for the tip-tracking as we also observed GFP-HSET tip-tracking when ATP was replaced by ADP (Supporting Information Fig. 1). GFP-HSET also interacted with the microtubule lattice (Fig. 1B), namely in a diffusive manner as evidenced by experiments performed at lower GFP-HSET concentration (Supporting Information Fig. 2).

Three lines of evidence support the idea that HSET tip-tracking is dependent on its interaction with hsEB1: (i) Using GFP-HSET in absence of hsEB1 we did not observe any tip-tracking while GFP-HSET interaction with the microtubule lattice prevailed (Fig. 1C). (ii) Examining the HSET amino acid sequence we found that HSET contains the SxIP-like motif SQLP. When we mutated the SQLP motif to SQNN to prevent its interaction with hsEB1 [Fong et al., 2009; Honnappa et al., 2009; Buey et al., 2012] the GFP-HSET-SQNN did not tip-track in presence of hsEB1, while the interaction with the microtubule lattice prevailed (Fig. 1D). (iii) We truncated the EB homology (EBH) domain of hsEB1 by removing 20 amino acids from the C-terminus (hsEB1ΔC), resulting in a construct, which is predicted to not interact with SxIP-like motifs [Honnappa et al., 2005, 2009; Montenegro Gouveia et al., 2010]. Again, we did not observe any tip-tracking, while GFP-HSET interaction with the microtubule lattice predominated (Fig. 1E). To confirm that the interaction between hsEB1 and HSET was not hsEB1 specific – but mediated by the conserved SxIP-like motif interaction with the EBH domain present in EB family proteins—we demonstrated GFP-HSET tip-tracking in presence of *D. melanogaster* EB1 (dmEB1) and *S. pombe* EB1 (Mal3) (Fig. 2).

To test whether the SxIP-like motif is indispensable for the tip-tracking of other kinesins-14 we *in vitro* reconstituted tip tracking of *D. melanogaster* Ncd and *S. pombe* Klp2 (Figs. 3A and 4A), two kinesin-14s which—in contrast to HSET—have been previously shown to tip-track *in vivo*, [Goshima et al., 2005; Janson et al., 2007]. Ncd in its N-terminus possesses the motif SRLP, which is expected to

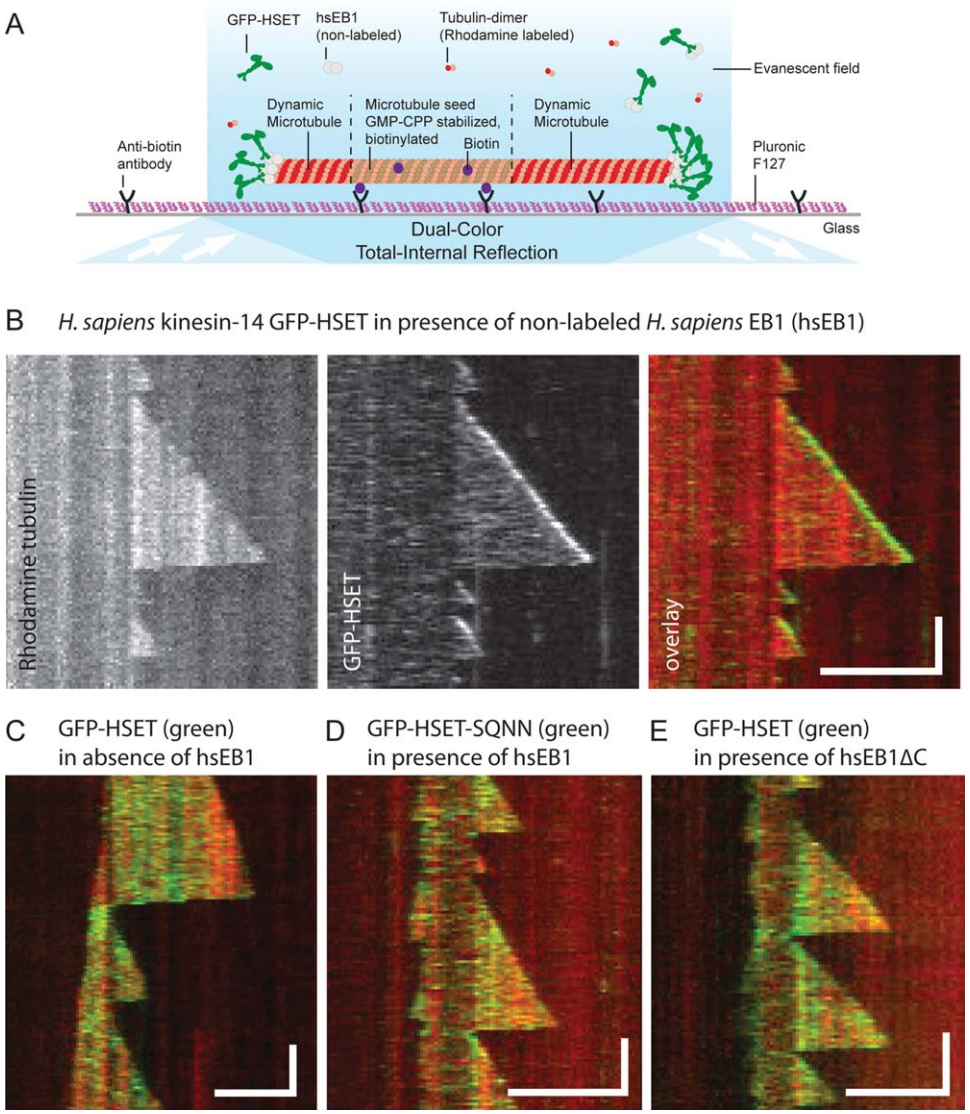

Fig. 1. *H. sapiens* kinesin-14 HSET microtubule tip-tracking is mediated by hsEB1. (**A**) Schematic of the *in vitro* GFP-kinesin-14 tip-tracking assay. (**B-E**) Typical multichannel kymographs showing GFP-kinesin-14 microtubule tip-tracking dependent on hsEB1: (**B**) GFP-HSET (15 nM) in presence of hsEB1 (22 nM), (**C**) GFP-HSET (15 nM) in absence of hsEB1 (**D**) GFP-HSET-SQNN (15 nM) in presence of hsEB1 (22 nM) (**E**) GFP-HSET (15 nM) in presence of hsEB1ΔC (1.3 μM). Microtubules are oriented with their plus-ends (identified as the faster growing microtubule ends) toward the right. Scale bars: horizontal, 5 μm; vertical, 1 min. All figures are scaled for optimal contrast, thus the intensities are not directly comparable.

interact with EB1. Consistent with this notion, we observed tip-tracking using a truncated Ncd construct (amino acids 1–349) containing the N-terminal SRLP motif but missing the C-terminal motor domain, showing that the motor domain was not necessary for Ncd tip-tracking (Supporting Information Fig. 3). Moreover, in accordance with the predictions of Buey et al., in vitro Ncd tip-tracking depended on the presence of the SRLP motif (Fig. 3C). GFP-Klp2 which possesses SHLP and SNIP, two motifs that are not expected to interact with EB1 [Buey et al., 2012], surprisingly tip-tracked dependent on Mal3, the EB1 homolog in *S. pombe* (Figs. 4A and 4B). By contrast to Ncd, the mutation of both SxIP-like motifs in Klp2 reduced but not abolished tip-tracking (Fig. 4C). Thus, the two SxIP-like motifs in the Klp2 N-terminal domain are not essential to mediate Mal3-dependent tip-tracking, which is in agreement with the recent finding that in vivo the simultaneous mutation of both motifs significantly reduces, but does not abrogate Klp2 tip-tracking [Mana-Capelli et al., 2012]. Our data show that for both Ncd and Klp2 the presence of EB1 is sufficient to induce tip-tracking.

Sequence comparison shows that the presence of an SxIP-like motif within the first 70 amino acids from the N-terminus is a common feature in kinesin-14 family proteins (Table I). The position of the motif at the very beginning of the amino acid chain, distal of the flexible kinesin-14 tail-domain, provides ideal accessibility for protein–protein interaction. The motif SRLP, which is prominent within

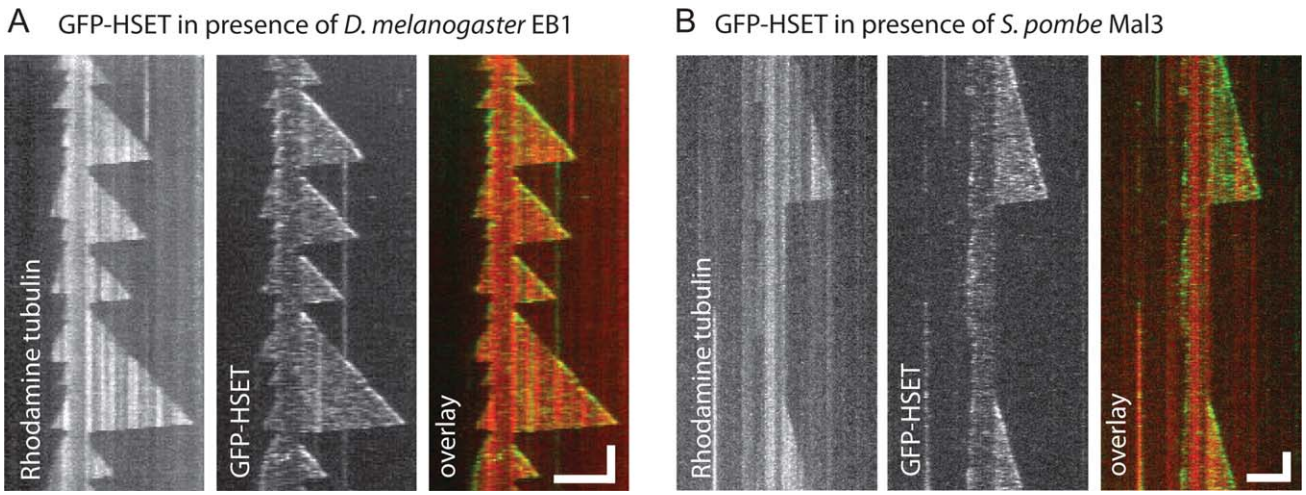

Fig. 2. *H. sapiens* kinesin-14 HSET microtubule tip-tracking in presence of *D. melanogaster* EB1 and *S. pombe* Mal3. Typical multichannel kymographs showing 15 nM GFP-HSET microtubule tip-tracking in presence of (**A**) 8 nM dmEB1 and (**B**) 6 nM Mal3. Microtubules are oriented with their plus-ends toward the right. Scale bars: horizontal, 5 μm; vertical, 1 min.

the kinesin-14 family, is an EB1 binding variant of the SxIP-like motif [Buey et al., 2012]. While human and its closest relative chimpanzee (*P. troglodytes*) kinesin-14 possesses the sequence SQLP, in other primates the sequence SRLP is conserved (Table I). The finding that the SRLP motif is highly conserved in kinesin-14s indicates that the

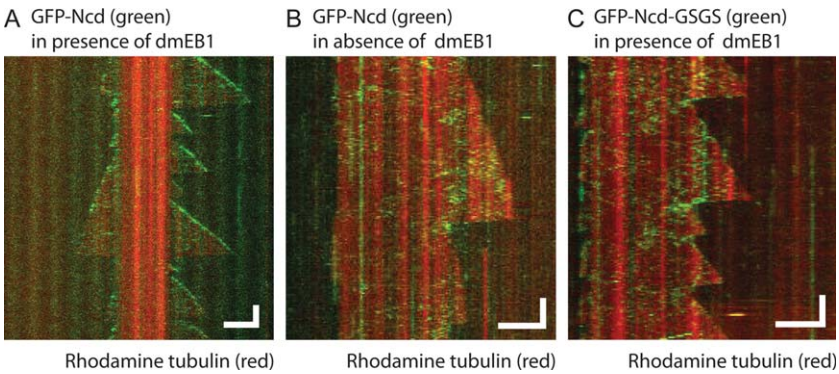

Fig. 3. *D. melanogaster* kinesin-14 Ncd in vitro tip-tracking is dependent on *D. melanogaster* EB1. This process requires the SRLP motif present in Ncd. Typical multichannel kymographs showing microtubule dynamics in presence of GFP-Ncd: (**A**) GFP-Ncd (10 nM) in presence of dmEB1 (8 nM), (**B**) GFP-Ncd (10 nM) in absence of EB1, (**C**) GFP-Ncd-GSGS (3 nM) in presence of dmEB1 (8 nM). Microtubules are oriented with their plus-ends toward the right. Scale bars: horizontal, 5 μm; vertical, 1 min.

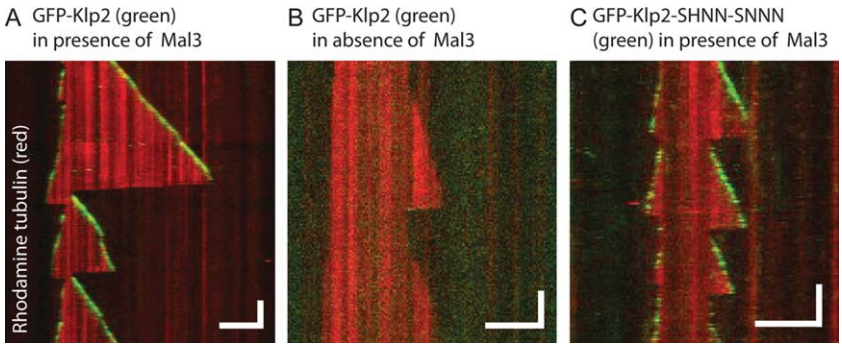

Fig. 4. *S. pombe* Klp2 tip-tracking is dependent on the *S. pombe* EB1 homolog Mal3. Typical multichannel kymographs of (**A**) 18 nM GFP-Klp2 tip-tracking dependent on 6 nM Mal3 and (**B**) 18 nM GFP-Klp2 in absence of Mal3. (**C**) GFP-Klp2-SHNN-SNNN (18 nM) in presence of Mal3 (6 nM). Microtubule plus-tips are oriented toward the right. Scale bars: horizontal, 5 μm; vertical, 1 min.

Table I. The SxIP-like Motif SRLP is Conserved within the Kinesin-14 Family

Kinesin-14	Sequence	UniProt Reference
HSET Human	1-MDPQRSPLLEVKGNIELKRPLIKAPSQLPLSGSRLKRRPDQMEDGLEPEKKRTRGLGATTKITTSHPRVP-70	Q9BW19
Kif1C Chimpanzee	1-MDPQRSPLLEVKGNIELKRPLIKAPSQLPLSGSRLKRRPDQMEDGLEPEKKRTRGLGATTKITTSHPRVP-70	H2QST6
Kif1C Gorilla	1-MDPQRSPLLEVKGNIELKRPLIKTPSRLPLSGSRLKRRPDQMEDGLEPEKKRTRGLGATTKITTSHPRVP-70	G3QDE3
Kif1C Orangatan	1-MD-PKSPLLEVKGNIELKRPLIKAPSRLPLSGSTLKRRPDQMEDGLEPEKKRTRGLGATTRITTSHPRVL-70	H2PIQ1
Kif1C Gibbon	1-MDLQRSPLLEVKGNIEPKRPLIKAPSRLPLSGSRLKRRPDQMEDGLEPEKKRTRGLGATTKITTSHPRVP-70	G1RA63
KIFC1 Rhesus	1-MDPQRSPLLEVKGNIELKRPRIKAPSRLPLSGSRLKRRPDQMEDGLEPEKKRTRGLGATTKITTSHPRVP-70	I0FRK2
Kif1C Marmorset	1-MDPQRSPLLELKGNIELKRPLIKAPSRLPLSGSRLKRRPDQMEDGLEPEKKRTRVLGATTKIATSRPRVP-70	F7IR24
KIFC1 Rat	1-MRGRGSRDTGTQSAAFASRPVRTTVDMQAQRAPLMEVKRNLELSTTLVKSSSRLPLPGSRLKRGPDQMED-70	Q5XI63
KIFC1 Mouse	1-MDVQAQRKGREGKRNVELKAALVKSSSRLPLSASSLKRGPDQMEDALEPAKKRTRVMGAVTKVDTSRPRG-70	Q5BJ94
KIFC1 Pig	1-METLRSPLLEVKGNIEVKRPLPKPPSRLPLSGSRLKRGPEQMEEALEPEKKRTRGLGTKIAPSRPRAALL-70	F1RZS7
KIFC1 Cow	1-MEPQRSPLLEVKGNVELKRPLAKAASRLPLSGRRLKRGPDQMEEALEPEKKRTRGLGTRVTTTHPRAAAL-70	A7MBA1
KIFC1 Tasman. devil	1-MEKGSKELKMPSDKASSSRLPVLGLGRKRRLDKENAPEPEKKRIRGTGTTIPMSCLKEATVATIPRAKKQ-70	G3VDU2
KIFC1 Opossum	1-MASCFLQKLSRMEKDNMELKMPPDKASSSQLPVLGLSAKRGLDKENVPEPKKKRARGPGTAATIAISHPR-70	F6XKS9
KIFC1 Chicken	1-MAAVGSGGSVGAAPGMAVVAPLPAPTSRLPVRRAAAKRAASGPQPAAPEQKRARSGTASSQPPGRAPLWA-70	A5HUJ1
KIFC1 Cynops	1-MNVDEKQPVAVMKSVSRLPVPSTLKTKRMRSNENMPVMEKKRLRLSSPDRVAQHRVPASIACTRPKPVAA-70	G4VV28
KIFC1 Octopus	1-MNGQRKVLADTANCSSKLPKLTPKLAKRKNSPNETEQVKKMRFQKPVSKIRTNLAPSSRLVNSQSIAGYN-70	D9D9T7
KIFC1 Crab	1-MSKLPSSASRHLHQPSRLRPPGSAMKRLGSDSAITSPQKKTRHSGDAEDTGAMARSQRLGGPRAAPGLSR-70	D9DBK9
KIFC1 Latimeria	1-MSEKVSSRLPVLKLGKKVLREENQQQRSLKRQCDTSPGHDLPKKKMVVSVVLKQSQAMAPIPRNPRGAGG-70	H3ADW2
KIFC1 Zebrafish	1-MNKENTSRLPVMSGKRAHTNSTDGEQQQPAQKKMRKVEVEPSQRFRPAASVAPPRRPVAVKAPVKPLRPT-70	Q7ZZ74
KLP15 C. elegans	1-MNVARRRSGLFRSTIGATPKITRGRAAAPSTKEANSTTIPRQSAPGGITIGAAARRPPSRLPTPTTPATG-70	P91400
KLP16 C. elegans	1-MNVARRRSGLFRSTIGAPPKATRGRAAAPPIKEADPATIPRQSAPGGITIGAAACRPPSRLPGATISATG-70	Q93366
XCTK2 Xenopus	1-MDSTDKKVQVASRLPVPPKRKYVSNDENQEQMQRKRLRSSLESELPAVRVAASIATSKPRAAPVAALPKP-70	Q5XGK6
Ncd Drosophila	1-MESRLPKPSGLKKPQMPIKTVLPTDRIRAGLGGGAAGAGAFNVNANQTYCGNLLPPLSRDLNNLPQVLER-70	P20480
Klp2 S. pombe	1-MEEEGHKSLTSHLPQSSSSLSQSREIAKEFTSNIPPPTIKTNSSSSNILKPRLSLQNEVNQLKPAKFPSK-70	Q9US03
ATK5 A. thaliana	1-MPLRNQNRAPLPSPNVKKEALSSIPFDKRRKETQGTGRRQVLSTVNRQDANSDVGSTEECGKVEFTKDEV-70	Q6NQ77

Sequence alignment of the N-terminal first 70 amino acids of kinesin-14 proteins from various species. Primates are listed in order of relatedness to humans. SxIP-like motifs are underlined. SRLP peptides are highlighted in green. Yellow highlighting denotes amino acids aberrant from the conserved SRLP, whose presence is predicted to promote interaction with EB1. Red highlighting denotes aberrant amino acids whose presence is predicted to inhibit interaction with EB1. SxIP-like motif sequence identifications and EB1 interaction predictions are based on Buey et al., [2012]. Bold letters in the first column indicate kinesin-14s, which to date have been observed to tip-track in vivo. UniProt references for the protein sequences are given in the last column.

preservation of the kinesin-14 tip-tracking ability is under high evolutionary pressure. The SRLP to SQLP mutation in human and chimpanzee does not contradict this finding, as the R to Q mutation preserves the tip-tracking ability.

It has been speculated that, analogous to *D. melanogaster* Ncd [Goshima et al., 2005], *A. thaliana* ATK5 tip-tracking observed *in vivo* could depend on EB1 [Ambrose et al., 2005]. ATK5 possesses an SxIP-like motif, which is predicted not to interact with EB1 (Table I). As in the case of Klp2 (Fig. 4C), this does not necessarily exclude a dependency of tip-tracking on EB1. ATK5 and Klp2 may recognize EB1 family proteins by interacting with a motif independent of SxIP. It is also conceivable that EB1 interactions with SxIP-like motifs permit a greater sequence variability compared to the canonical 'SxIP' than described in the recent literature [Buey et al., 2012; Jiang et al., 2012]. Therefore Klp2, ATK5 and other kinesin-14s might recognize clusters of amino acids that are currently not detectable in the protein sequences.

Here, we showed that EB1 promotes tip-tracking of GFP-HSET and GFP-Klp2. Why, in contrast to Klp2, has HSET tip-tracking not been observed in vivo [Cai et al., 2009]? When comparing the affinities of GFP-HSET and GFP-Klp2 for EB1 we did not observe any significant difference (Fig. 5). However, in our experiments we found that GFP-HSET interacted more strongly than GFP-Klp2 with the microtubule lattice (compare Figs. 1B and 4A). Under certain conditions, e.g., when the concentration of GFP-HSET was higher than presented in Fig. 1, the strong

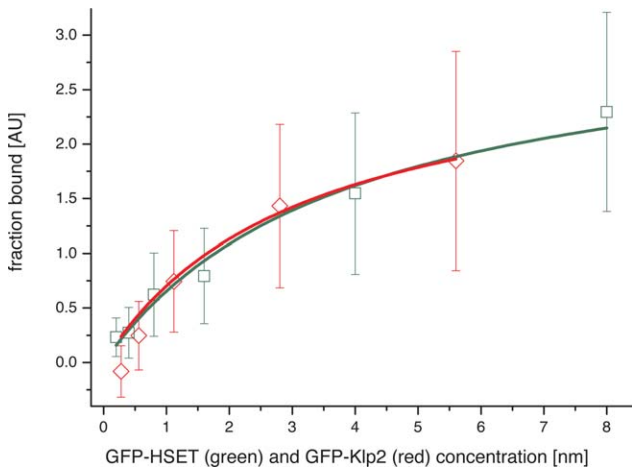

Fig. 5. HSET and Klp2 affinity for hsEB1. Dissociation constants of GFP-HSET and GFP-Klp2 binding to hsEB1 ($K_D^{HSET} = 4 \pm 1$ nM and $K_D^{Klp2} = 3 \pm 1$ nM) are estimated from the fit assuming a noncooperative binding model. Arbitrary units (AU) on the *y*-axis represent background-subtracted fluorescence intensities of GFP-HSET (green) and GFP-Klp2 (red) binding to hsEB1, unspecifically adsorbed to a glass coverslip. The fluorescence signal from a 10 µm x 10 µm coverslip area was measured by TIRF microscopy. Data are presented as an average value of 10 measurements with error bars indicating the standard deviation.

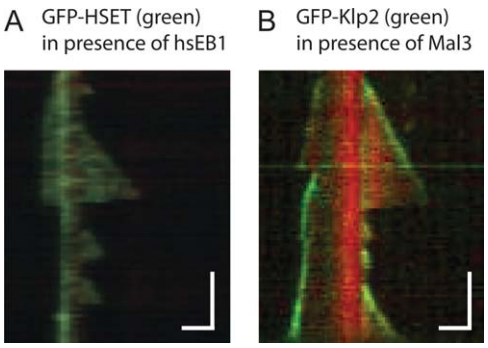

Fig. 6. *H. sapiens* kinesin-14 HSET tip-tracking is obscured by its interaction with the microtubule lattice at high HSET concentration. (**A**) Typical multichannel kymograph showing 75 nM GFP-HSET (green) microtubule (red) tip-tracking in presence of 22 nM hsEB1. GFP-HSET is 5 times more concentrated compared to Figure 1, while hsEB1 concentration is the same. (**B**) Typical multichannel kymograph showing 100 nM GFP-Klp2 (green) microtubule (red) tip-tracking in presence of 6 nM Mal3. All figures are scaled for optimal contrast, thus the intensities are not directly comparable. Microtubules are oriented with their plus-ends toward the right. Scale bars: horizontal, 2 µm; vertical, 1 min.

lattice interaction did indeed completely obscure tip-tracking of GFP-HSET (Fig. 6A). Under similar conditions, tip-tracking of GFP-Klp2 was still clearly observable (Fig. 6B). We thus argue that HSET tip-tracking in vivo might be obscured by the pronounced interaction of HSET with the microtubule lattice. In consequence, EB1-dependent microtubule tip-tracking of HSET may also be present in vivo and may have functional effects. Mutating the SxIP-like motif of HSET in vivo, such that its specific interaction with hsEB1 is inhibited, would open up a route to explicitly study the role of tip-associated HSET while keeping microtubule bundling and sliding conferred by HSET-interaction with the microtubule lattice intact.

In summary, we here demonstrate microtubule tip-tracking of human kinesin-14 HSET and show that EB1 is sufficient to induce this dynamic microtubule end localization. Furthermore, we provide evidence that SxIP-like motif SRLP is conserved in the N-terminal region of molecular motors of the kinesin-14 family. It is likely that kinesin-14 tip-tracking dependent on EB family proteins is conserved, potentially playing important functional roles yet to be elucidated in various species including human.

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