A biosensor of local kinesin activity reveals roles of PKC and EB1 in KIF17 activation

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We showed previously that the kinesin-2 motor KIF17 regulates microtubule (MT) dynamics and organization to promote epithelial differentiation. How KIF17 activity is regulated during this process remains unclear. Several kinesins, including KIF17, adopt compact and extended conformations that reflect autoinhibited and active states, respectively. We designed biosensors of KIF17 to monitor its activity directly in single cells using fluorescence lifetime imaging to detect Förster resonance energy transfer. Lifetime data are mapped on a phasor plot, allowing us to resolve populations of active and inactive motors in individual cells. Using this biosensor, we demonstrate that PKC contributes to the activation of KIF17 and that this is required for KIF17 to stabilize MTs in epithelia. Furthermore, we show that EB1 recruits KIF17 to dynamic MTs, enabling its accumulation at MT ends and thus promoting MT stabilization at discrete cellular domains.

Introduction

Modulation of microtubule (MT) dynamics and reorganization of the MT cytoskeleton are key events accompanying cellular morphogenesis during differentiation and tissue remodeling (Gierke and Wittmann, 2012). This change in cytoskeletal organization and dynamics is often mediated by an evolutionarily conserved mechanism involving capture of MT plus ends by cortical factors that favor local MT stabilization (Gundersen, 2002; Wu et al., 2006). We showed that, in epithelial cells, the kinesin-2 family motor KIF17 associates with MT plus ends via an interaction with the EB1 (end-binding protein 1). We also demonstrated that KIF17 dampens MT dynamics, contributes to MT stabilization, and is necessary for polarization of epithelial cells in 3D matrices. We proposed that active KIF17 could participate in regulating interactions of MT plus ends and cortical proteins during MT capture and stabilization (Jaulin and Kreitzer, 2010). However, how KIF17 activity is regulated temporally and spatially to contribute to MT dynamics and epithelial polarization is not known.

Kinesins are MT-stimulated mechanoenzymes that use ATP hydrolysis to generate motile forces (Schiwa and Woehlke, 2003; Vale, 2003). Several kinesins, including KIF17, are regulated by an autoinhibitory mechanism wherein the motor and tail domains interact, resulting in reduced MT binding and/or ADP release (Coy et al., 1999; Hackney and Stock, 2000; Imanishi et al., 2006; Dietrich et al., 2008; Verhey and Hammond, 2009; Hammond et al., 2010; Jaulin and Kreitzer, 2010). To understand how KIF17 is regulated in epithelial cells for MT stabilization, we designed kinesin biosensor constructs that are monitored using fluorescence lifetime imaging microscopy (FLIM). These biosensors provide a readout of kinesin conformation based on measurements of intramolecular Förster resonance energy transfer (FRET) efficiency (Wallrabe and Periasamy, 2005); inactive motors in a compact conformation generate FRET, whereas active motors in an extended conformation do not. FRET-based, sensitized emission approaches have been used in live cells to detect kinesin-1 and kinesin-3 in compact and extended conformations (Cai et al., 2007; Hammond et al., 2009). However, quantitative determination of active and inactive kinesin populations and their spatial distributions cannot be resolved with this approach. By comparison, FLIM improves sensitivity, is quantitative, and allows independent determinations of FRET efficiency and the fraction of interacting donor molecules (Piston and Kremers, 2007; Padilla-Parra and Tramier, 2012). Here, we apply phasor analysis to FLIM (Clayton et al., 2004; Redford and Clegg, 2005; Caiolfa et al., 2007; Digman et al., 2008), allowing us to localize active and inactive kinesin populations in single cells for the first time across large datasets.

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Abbreviations used in this paper: EmGFP, Emerald GFP; FLIM, fluorescence lifetime imaging microscopy; FRET, Förster resonance energy transfer; mCh, mCherry; MT, microtubule; NZ, nocodazole; OA, okadaic acid.

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Using a KIF17 biosensor, we provide direct evidence that KIF17 conformation and activity are regulated by EB1 and PKC. Our data suggest that PKC activates KIF17 for binding to dynamic MTs and that EB1 promotes KIF17 accumulation in an active form at the ends of dynamic MTs. Both EB1 and active PKC affect KIF17 conformation in cells and are likely to contribute to selective MT stabilization by KIF17 in epithelia. The data presented here provide the first direct visualization of extended, active and compact, inactive kinesin populations in living cells and demonstrate that conformational biosensors monitored by FLIM, combined with phasor analysis of lifetime data, provide a significant technical advance over current approaches to study kinesin regulation in living cells.

Results and discussion

Active KIF17 in an extended conformation localizes at the cell periphery in MDCK epithelial cells

We and others have shown that KIF17 undergoes a salt-dependent change in conformation in vitro (Imanishi et al., 2006; Hammond et al., 2010; Jaulin and Kreitzer, 2010). To detect KIF17 conformations in cells directly, we designed intramolecular FRET expression constructs encoding full-length KIF17 tagged with mCherry (mCh) and Emerald GFP (EmGFP) at N and C termini, respectively. We used FLIM to measure the decay in EmGFP lifetime caused by quenching by mCh in live MDCK cells (Bastiaens and Squire, 1999). In all experiments, donor lifetimes in cells expressing FRET constructs (mCh-KIF17-EmGFP) were referenced to cells expressing donor alone (KIF17-EmGFP). Only cells with low to medium expression levels were chosen for analysis to avoid artifacts caused by overexpression. Lifetime data were analyzed using the phasor approach. This method is amenable to rapid analysis of a large number of cells and reports the localization of kinesin populations in compact and extended states in the cell as well as the relative amount of each population (see Materials and methods and Fig. S1 [B and C] for details).

We first determined that N- and C-terminal tags did not interfere with KIF17 function by testing whether FRET constructs stabilized MTs when expressed in MDCK cells, as described previously for the constitutively active, extended conformation hinge mutant GFP-KIF17<sup>G754E</sup> (Jaulin and Kreitzer, 2010). In cells expressing mCh-KIF17-EmGFP, KIF17-EmGFP, GFP-KIF17, or mCh-KIF17<sup>G754E</sup>-EmGFP, we observed an increase in nocodazole (NZ)-resistant, posttranslationally modified (de tyrosinated or acetylated) stable MTs (Fig. 1, A and E; and not depicted). Thus, fluorescently tagged wild-type and mutant KIF17 are active for MT stabilization, demonstrating that these constructs are functional in cells.

As shown previously for GFP-KIF17 (Jaulin and Kreitzer, 2010), exogenously expressed mCh-KIF17-EmGFP appeared primarily as diffuse cytoplasmic fluorescence (Fig. 1 B, inset grayscale image), suggesting that the majority of KIF17 is in a compact, inactive conformation as previously described (Hammond et al., 2010). Using FLIM, we measured a mean FRET efficiency (E<sub>AV</sub>) for mCh-KIF17-EmGFP of 6.8 ± 0.2% (Fig. 1 D and Table 1). However, this mean could reflect a mixture of both compact (FRET signal) and extended (non-FRET) populations of mCh-KIF17-EmGFP. To discriminate between these two populations, we mapped the spatial distribution of FRET and non-FRET species in cells using phasor analysis. Based on our FRET confidence limit (see Materials and methods), pixels in the image with FRET,<sub>eff</sub> ≤ 7% represent KIF17 in an extended conformation, whereas pixels with FRET,<sub>eff</sub> = 7–21% represent KIF17 in a compact form (Fig. 1 B, phasor plot). Phasor analysis revealed that 64.9 ± 2.0% of mCh-KIF17-EmGFP is in a compact state (Fig. 1 D). This pool of mCh-KIF17-EmGFP localizes throughout the cytoplasm but does not generally extend to the cell periphery (Fig. 1 B, green mask). A smaller but significant proportion (25.9 ± 1.4%) of mCh-KIF17-EmGFP was in the extended conformation (Fig. 1 D) and localized prominently near the cell periphery (Fig. 1 B, red mask). The cortical distribution of extended KIF17 is consistent with the EB1-dependent localization of endogenous protein to MT plus ends and a role in plus-end-mediated MT stabilization (Jaulin and Kreitzer, 2010). It is likely that some extended KIF17 is also located on more central MTs, but this pool is obscured in our analysis by the greater cell depth and larger fraction of motor in the compact form in these regions. Extended KIF17 was also detected above/below nuclei, where the cytoplasmic depth is narrow, and compact KIF17 did not obscure detection of an extended motor.

To confirm that the KIF17 we detect in an extended conformation represents the population of KIF17 on MTs, we permeabilized living cells in the presence of AMP-PNP, a nonhydrolyzable ATP analogue that recruits kinesins to MTs (Laske and Brady, 1985). Under these conditions, mCh-KIF17-EmGFP E<sub>AV</sub> decreased 65%, with a corresponding 2.3-fold increase in KIF17 in an extended form (Fig. S1, D and E; and Table 1), consistent with previous studies (Hammond et al., 2010). Thus, KIF17 in an open conformation by FRET represents the population of motor on MTs.

To verify that different KIF17 conformations detected with FRET reflect motor activity, we next analyzed the constitutively active hinge mutant mCh-KIF17<sup>G754E</sup>-EmGFP. The FRET efficiency of mCh-KIF17<sup>G754E</sup>-EmGFP was 66% less than that of mCh-KIF17-EmGFP (E<sub>AV</sub> = 2.3 ± 0.6%; Fig. 1 D and Table 1). Phasor analysis showed that 70.1 ± 4.0% of KIF17<sup>G754E</sup> is in an extended conformation, a 2.7-fold increase as compared with KIF17; only 13.8 ± 2.0% of KIF17<sup>G754E</sup> is detected in a compact conformation (Fig. 1 D and Table 1). Conformationally extended mCh-KIF17<sup>G754E</sup>-EmGFP localized throughout the cell and accumulated at MT plus ends in cell protrusions (Fig. 1 C, red mask and inset grayscale image), as shown previously for GFP-KIF17<sup>G754E</sup> (Jaulin and Kreitzer, 2010). Some FRET was detected in these protrusions (Fig. 1 C, green mask) and likely arises from crowding-induced intermolecular interactions rather than motors in a folded state, as suggested previously for kinesin-1 (Cai et al., 2007).

Further evidence that KIF17 conformation reflects its activity comes from data showing that KIF17 motor and tail domains interact and that full-length KIF17 is not efficient for MT-based motility but that removal of the C-terminal tail or mutation of the hinge produces a motor that moves effectively on MTs (Imanishi et al., 2006; Hammond et al., 2010; Jaulin...
Figure 1. **Localization of active and inactive populations of KIF17 in MDCK cells.** (A) Immunostaining of tyrosinated and detyrosinated tubulin in cells microinjected with mCh-KIF17-EmGFP (outlined cells and inset) and treated for 45 min with 33 µM NZ. (B) Representative FLIM phasor analysis of mCh-KIF17-EmGFP in cells. Fluorescence image (inset) and the distribution of KIF17 in extended, active (red mask on image and red circle on phasor plot, FRETeff < 7%) and compact, inactive (green mask on image and green circle on phasor plot, FRETeff = 7–21%) forms determined from measured lifetimes. Inset on the phasor plot shows analysis of mCh-KIF17G754E-EmGFP. (C) Representative FLIM phasor analysis of mCh-KIF17G754E-EmGFP in cells as in B. (D) Box-whisker plots showing the distribution of FRETeff and populations of active and inactive mCh-KIF17-EmGFP and mCh-KIF17G754E-EmGFP in in MDCK cells. Data represent the indicated number of cells (n) obtained from three or more independent experiments ± SEM. Box-whisker plots show minimum, 25th percentile, median, 75th percentile, maximum, and mean FRET values. (E) Quantification of immunoblots probed for detyrosinated (Glu) and acetylated (Ace) tubulin in control or mCh-KIF17-EmGFP transfected cells. Ratio of tubulin/actin was normalized to 1 ± SD in controls. Data are derived from five experiments. Bars, 20 μm.
and Kreitzer, 2010). Similarly, recombinant OSM-3, the Caenorhabditis elegans homologue of KIF17, has little to no motility in vitro, but both a hinge mutant and C-terminally truncated OSM-3 move efficiently on MTs (Imanishi et al., 2006; Hammond et al., 2010; Jaulin and Kreitzer, 2010). Here, using purified recombinant protein, we found that the KIF17 tail domain (amino acids 850–1,029) ablates MT gliding and inhibits MT-stimulated ATPase activity in vitro of C-terminally truncated KIF17 (amino acids 1–490, KIF17M-490; Fig. S1 A and Table 2). In these assays, KIF17M-490 had a maximal MT-stimulated ATPase activity of C-terminally truncated KIF17 (amino acids 1–490, KIF17M-490; Fig. S1 A and Table 2). In these assays, KIF17M-490 had a maximal MT-stimulated ATPase activity of C-terminally truncated KIF17 (amino acids 1–490, KIF17M-490; Fig. S1 A and Table 2). In these assays, KIF17M-490 had a maximal MT-stimulated ATPase activity of C-terminally truncated KIF17 (amino acids 1–490, KIF17M-490; Fig. S1 A and Table 2). In these assays, KIF17M-490 had a maximal MT-stimulated ATPase activity of C-terminally truncated KIF17 (amino acids 1–490, KIF17M-490; Fig. S1 A and Table 2).

Because KIF17 may act through this pathway (Jaulin and Kreitzer, 2010), we tested whether PKC affects KIF17 conformation and activity for MT stabilization. We treated MDCK cells expressing mCh-KIF17-EmGFP with the PKC inhibitor BIM-1 (bisindolylmaleimide I; 20 µM) for 1 h (Fig. 2); at this concentration, classical PKCs α and β and novel PKCs δ and ε are likely inhibited (Martiny-Baron et al., 1993).

BIM-1 induced a 20% increase over controls in $E_{AV}$ of mCh-KIF17-EmGFP and a 41% decrease in the extended population of KIF17 (Fig. 2, A and B; and Table 1). Furthermore, BIM-1–treated cells expressing mCh-KIF17-EmGFP (or KIF17-EmGFP) failed to generate NZ-resistant, stable MTs (Fig. 2 C). These data implicate PKC in regulating KIF17 conformation and thus its ability to interact with and stabilize MTs in cells. Inhibition of PKC could alternatively prevent generation of stable MTs by affecting downstream components in the MT stabilization pathway (e.g., GSK3-δ), as suggested in fibroblasts (Eng et al., 2006); this could inhibit KIF17 activity indirectly by affecting its binding to and unfolding on MTs. However, BIM-1 alone had no effect on levels of posttranslationally modified, stable MTs in MDCK cells (unpublished data). Furthermore, pretreatment of cells with BIM-1 did not affect the localization of the extended mutant GFP-KIF17G754E at MT ends in cell protrusions (Fig. 2 D) or its ability to stabilize MTs (not depicted). Considered together, these data support a model in which active PKC releases KIF17 autoinhibition, promoting binding to and stabilization of MTs.

Table 1. Summary of FRET efficiencies and percentage of active, extended KIF17 measured under all conditions described

<table>
<thead>
<tr>
<th>Expressions</th>
<th>Treatments</th>
<th>$E_{AV}$</th>
<th>P-value</th>
<th>Active pool</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCh-KIF17-EmGFP</td>
<td>Untreated</td>
<td>6.8 ± 0.2</td>
<td>NA</td>
<td>25.9 ± 1.4</td>
<td>NA</td>
</tr>
<tr>
<td>mCh-KIF17-EmGFP</td>
<td>1 mM AMP-PNP</td>
<td>2.4 ± 0.9</td>
<td>&lt;0.001</td>
<td>58.4 ± 4.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mCh-KIF172035EmGFP</td>
<td>Untreated</td>
<td>2.3 ± 0.6</td>
<td>&lt;0.001</td>
<td>70.1 ± 4.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mCh-KIF17-EmGFP</td>
<td>33 µM NZ</td>
<td>7.0 ± 0.4</td>
<td>&gt;0.5</td>
<td>25.9 ± 3.7</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>mCh-KIF17-EmGFP</td>
<td>Cold + 33 µM NZ</td>
<td>9.7 ± 0.5</td>
<td>&lt;0.001</td>
<td>13.2 ± 3.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mCh-KIF17-EmGFP</td>
<td>100 nM OA</td>
<td>7.8 ± 0.4</td>
<td>&lt;0.05</td>
<td>15.9 ± 2.6</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>mCh-KIF17-EmGFP</td>
<td>10 µM taxol</td>
<td>9.3 ± 0.6</td>
<td>&lt;0.001</td>
<td>11.4 ± 2.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mCh-KIF17-EmGFP</td>
<td>20 µM BIM-1</td>
<td>8.5 ± 0.5</td>
<td>&lt;0.001</td>
<td>16.4 ± 1.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mCh-KIF17-EmGFP + Myc-EB1</td>
<td>Untreated</td>
<td>5.4 ± 0.4</td>
<td>&lt;0.001</td>
<td>40.2 ± 4.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mCh-KIF17-EmGFP + Myc-EB1</td>
<td>1 mM AMP-PNP</td>
<td>6.7 ± 0.5</td>
<td>&lt;0.005</td>
<td>24.2 ± 3.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mCh-KIF17-EmGFP + Myc-EB1</td>
<td>Cold + 33 µM NZ</td>
<td>8.5 ± 0.6</td>
<td>&lt;0.001</td>
<td>16.3 ± 1.8</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

$^{a}$Value distribution as compared with mCh-KIF17-EmGFP using a two-tailed Student’s t test.

NA, not applicable.

Notes:

- $k_{cat}$ refers to the rate of ATP hydrolysis per kinesin dimer.
- $K_{0.5, MT}$ refers to the concentration of polymerized tubulin to half-maximally stimulate ATPase activity. Errors are ± SEM.

PKC activity is necessary to relieve KIF17 autoinhibition and activate it for MT stabilization

PKC family members have been implicated in regulation of MT dynamics and polarization of several cell types and act in the cortical MT capture and stabilization pathway (Kabir et al., 2001; Fan et al., 2004; Ruiz-Canada et al., 2004; Eng et al., 2006).

Table 2. ATPase properties of KIF17M-490

<table>
<thead>
<tr>
<th>Constructs</th>
<th>ATPase activity</th>
<th>Gliding</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$</td>
<td>$K_{0.5, MT}$</td>
<td>Velocity</td>
<td>Motile MT</td>
<td>No. of MT observed</td>
<td></td>
</tr>
<tr>
<td>KIF17 M-490</td>
<td>13.6 ± 1.8</td>
<td>1.3 ± 0.1</td>
<td>0.47 ± 0.02</td>
<td>11.6 ± 0.9</td>
<td>247</td>
<td></td>
</tr>
<tr>
<td>KIF17 M-490 + KIF17-tail</td>
<td>3.9 ± 0.7</td>
<td>0.4 ± 0.3</td>
<td>ND</td>
<td>ND</td>
<td>252</td>
<td></td>
</tr>
<tr>
<td>KIF17 M-490 + EB1</td>
<td>16.6 ± 1.2</td>
<td>0.7 ± 0.3</td>
<td>0.51 ± 0.03</td>
<td>16.6 ± 1.3</td>
<td>251</td>
<td></td>
</tr>
</tbody>
</table>

ATPase activity of KIF17M-490 in the absence and presence of EB1 or EB1. Data (Fig. S1 A) were fit to a Michaelis–Menten equation to determine $k_{cat}$ and $K_{0.5, MT}$. Data represent the mean of nine independent experiments at three different MT concentrations ± SEM. $k_{cat}$ refers to the rate of ATP hydrolysis per kinesin dimer. $K_{0.5, MT}$ refers to the concentration of polymerized tubulin to half-maximally stimulate ATPase activity. Errors are ± SEM.
contains several predicted PKC phosphorylation sites and thus could be regulated directly by PKC. Alternatively, PKC could affect KIF17 binding to a cargo that would activate the motor in cells (Verhey and Hammond, 2009).

**EB1 keeps KIF17 in an active form at MT plus ends in MDCK cells**

We showed previously that localization of endogenous KIF17 to MT plus ends in epithelial cells, where it could participate in cortical MT stabilization, is dependent on EB1 but that mutant KIF17<sup>G754E</sup> accumulates at plus ends and stabilizes MTs independent of EB1 (Jaulin and Kreitzer, 2010). This led us to speculate that EB1 binding to KIF17 at MT ends could either activate the motor for MT stabilization or enhance its accumulation in an active form at MT ends. To test this directly, we coexpressed mCh-KIF17-EmGFP and Myc-EB1 in MDCK cells and analyzed KIF17 confirmation with FLIM (Fig. 3). In these experiments, EB1 coexpression resulted in a 20% decrease in E<sub>AV</sub> as...
Figure 3. Coexpression of EB1 increases the pool of extended, active KIF17 in MDCK cells. (A) Fluorescence images of mCh-KIF17-EmGFP expressed alone or with Myc-EB1. Red mask shows the localization of active mCh-KIF17-EmGFP. Bar, 20 µm. (B and D) Box–whisker plots showing the distribution of FRETeff under each experimental condition. (C and E) Box–whisker plots showing the fraction of active KIF17 under each experimental condition. Data were obtained from at least three independent experiments ± SEM. Box–whisker plots show minimum, 25th percentile, median, 75th percentile, maximum, and mean FRET values.
compared with control cells expressing KIF17 alone (Fig. 3 B and Table 1). Phasor analysis showed this change in FRET efficiency corresponded to a 55% increase in the population of extended KIF17 when EB1 was coexpressed (Fig. 3 C and Table 1).

When cells coexpressing EB1 and KIF17 are treated with 33 μM NZ for 45 min to depolymerize dynamic MTs (note that some stable, modified MTs remain even in un.injected cells; Fig. 1 A), the population of extended KIF17 decreases to levels measured in untreated cells expressing KIF17 alone (Fig. 3, D and E; and Table 1). In contrast, when cells are preincubated on ice for 20 min before rewarmin in NZ for 45 min, a treatment that results in loss of both stable and dynamic MTs in MDCK cells (Kreitzer et al., 2003), we measure no difference in E_avg or the extended population of KIF17 when it is expressed alone or coexpressed with EB1 (Fig. 3, D and E). These data indicate that the EB1-mediated increase in conformationally extended KIF17 is dependent on dynamic MTs and that, in the absence of MTs, EB1 cannot recruit/retain KIF17 on MTs and thus cannot relieve the autoinhibited state.

In ATPase assays in vitro, EB1 did not affect k_cat of KIF17-M-490 significantly (P > 0.1) but decreased K_0.5,MT of KIF17-M-490 approximately twofold (P < 0.001; Fig. S1 B and Table 2), similar to what was shown for kinesin-7 (Tea2) in yeast (Browning and Hackney, 2005). In MT-gliding assays, EB1 similarly induced a 70% increase in the number of motile MTs without changing KIF17-M-490 velocity significantly. Collectively, these data suggest EB1 may decrease the off rate of KIF17 from MTs and thus retain KIF17 in an extended conformation at dynamic MT ends, promoting MT stabilization.

**Active KIF17 is on stable MTs in MDCK cells**

The constitutively active, extended mutant GFP-KIF17_G754E accumulates in large puncta at MT plus ends near the cell cortex in epithelial cells. This localization is observed even in cells treated with NZ to depolymerize dynamic MTs (Jaulin and Kreitzer, 2010), suggesting that active KIF17 is on stable MTs.

To determine whether extended KIF17 localizes on a specific subset of MTs, we analyzed mCh-KIF17-EmGFP by FLIM when only dynamic MTs or both dynamic and stable MTs were depolymerized. Depolymerization of dynamic MTs had no effect on E_avg or on the fraction of extended conformation mCh-KIF17-EmGFP as compared with untreated controls (Fig. 4, A and B). However, when both dynamic and stable MTs were depolymerized, we measured a 30% increase in E_avg accompanied by a 43% decrease in the extended population of KIF17 when compared with control untreated cells (Fig. 4, A and B). To break down stable, modified MTs selectively, we treated cells expressing mCh-KIF17-EmGFP with 100 nM okadaic acid (OA) for 90 min (Fig. S2 A; Gurland and Gundersen, 1993). OA induced a 42% decrease in the extended population of KIF17 to 15.9 ± 2.6% (Fig. S2, B and C), similar to what we observed in cells treated with cold and NZ. Although OA depolymerizes stable, modified MTs preferentially in MDCK cells, we cannot yet exclude the possibility that it affects KIF17 conformation and activity by modifying phosphorylation of the kinesin directly or of another factor that modulates KIF17 activity in our cells.

These experiments show that extended KIF17 resides primarily on stable MTs in MDCK cells. As such, we reasoned that stabilizing MTs with taxol (10 μM for 1 h) should lead to an increase in the pool of active, extended mCh-KIF17-EmGFP. Surprisingly, we instead measured a 37% increase in E_avg and a corresponding 56% decrease in the extended pool of KIF17 after taxol treatment (Fig. 4, A and B), similar to what was observed in cells treated with cold and NZ. Although OA depolymerizes stable, modified MTs preferentially in MDCK cells, we cannot yet exclude the possibility that it affects KIF17 conformation and activity by modifying phosphorylation of the kinesin directly or of another factor that modulates KIF17 activity in our cells.

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where it can participate in MT stabilization and then remain on stable MTs. Consistent with this idea, when living cells are permeabilized in the presence of AMP-PNP, we detect KIF17-EmGFP on both unmodified and posttranslationally modified MTs (Fig. S3 B). Because the majority of extended conformation (Fig. S3 A). Because KIF17G754E remains on MT plus ends in the absence of EB1 (Jaulin and Kreitzer, 2010), it is not likely that loss of EB1 from MTs in taxol (unpublished data) causes the observed changes in KIF17 activity and localization. These results suggest KIF17 uses dynamic MTs to reach MT plus ends where it can participate in MT stabilization and then remain on stable MTs. Consistent with this idea, when living cells are permeabilized in the presence of AMP-PNP, we detect KIF17-EmGFP on both unmodified and posttranslationally modified MTs (Fig. S3 B). Because the majority of extended conformation
KIF17 resides on stable MTs and because we measure no change in $E_{av}$ or extended mCh-KIF17-EmGFP when dynamic MTs are depolymerized selectively, we speculate that the pool of KIF17 being activated on dynamic MTs is small and, thus, below our detection limit with FRET analysis.

Together, our data support a model (Fig. 5) wherein KIF17 is activated through a pathway involving PKC that induces a conformational change sufficient for binding to MTs. Next, motile KIF17 interacts with EB1 at dynamic MT plus ends, inducing accumulation of activated motor at these sites. Activated KIF17 at MT plus ends could potentially interact directly, or through a cargo, with cortical factors involved in regulating MT stability, forming a “stable” bridge between MTs and the cortex. Alternatively, KIF17 could protect MT ends independent of cargo or cortical factors. Indeed, we find that the KIF17 motor domain is sufficient to protect MTs from depolymerization in vitro (Acharya et al., 2013). Further studies exploring these important questions are underway. The results of this work provide novel mechanistic insight into how KIF17 participates in MT stabilization in epithelial cells. In addition, these studies demonstrate that kinesin biosensors, monitored by FLIM in living cells and analyzed on phasor plots, can be applied widely to study kinesin regulation and to identify physiologically relevant factors that modify kinesin conformation and activity in vivo.

Materials and methods

Cell culture, transfection, and microinjection

MDCK cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (4.5 g/liter glucose) with 5% FBS and 20 mM HEPES, pH 7.2. MDCK cells were seeded on sterilized coverslips and used at 80% confluence 2–3 d after plating. Transfections (5 µg DNA) were performed using a nucleofector (Amaxa; Lonza) as recommended. Microinjection was performed essentially as described previously (Jaulin et al., 2007). 5–20 µg/ml cDNAs in HKCl (10 mM Hepes and 140 mM KCl, pH 7.4) were pressure injected using a micromanipulator (MMO-202ND; Narishige). Cells were incubated at 37°C for 60–90 min to allow for expression of cDNAs. NZ and taxol were purchased from Sigma-Aldrich. OA was obtained from EMD Millipore. For FLIM, we analyzed only cells expressing the biosensor at low to medium levels (less than or equal to mean expression – 0.3% and extended population = 22.4 ± 2.4%; mean/median expression, $E_{av} = 7.2 ± 0.2$% and extended population = 22.2 ± 1.9%). Immuno-fluorescence staining showed that biosensor expression is four to five times above levels of endogenous KIF17.

Fluorescence lifetime imaging

For all of these (≤30 amino acids), we saw no loss of MT association or fluorescence lifetime. For all of these constructs, we saw no loss of MT association or fluorescence lifetime. Therefore, we also tested constructs with long linkers in our experiments. With conformational regulation of the protein (Hammond et al., 2010), that large epitope tags connected to kinesins via short linkers to the N and C termini of kinesin, respectively, with a 10–amino acid linker between the tags and the kinesin. A previous study in COS cells suggested that large epitope tags connected to kinesins via short linkers to the N (4 amino acids) or C (10 amino acids) termini of KIF17 could interfere with conformational regulation of the protein (Hammond et al., 2010). Therefore, we also tested constructs with long linkers in our experiments. For all of these (≤30 amino acids), we saw no loss of MT association or significant difference in KIF17 activity by FLIM.

Constrasts and protein purification

Recombinant, functional mCh-KIF17-EmGFP (or any version of full-length KIF17) could not be purified in sufficient quantities in any host system tested. Therefore, we instead generated a Terminally truncated protein encoding KIF17M-490 (amino acids 1–490) containing the motor, neck, and first coiled-coil domains of KIF17 for analysis in vitro. The in vitro activity of KIF17M-490 was identical to a version of the protein lacking the first coiled coil (KIF17M-370). KIF17M-490 was amplified by PCR from full-length human KIF17 in Jaulin and Kreitzer (2010) and cloned into p201DONR (Invitrogen) as described for biosensor constructs. The forward primer user to generate KIF17M-490 was: 453A FRET biosensor detects local activation of KIF17 Espenel et al.

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were removed by passing cycled tubulin over DEAE-Sephadex. Tubulin purity was analyzed by SDS-PAGE and Coomassie blue staining. After two cycles, tubulin in PEM-GTP$_{inh}$ was snap frozen in single-use aliquots and stored at $-80^\circ$C. Before use, an additional cycle of cold disassembly and centrifugation at 4°C was performed to remove protein aggregates.

For in vitro assays, 25 μM DEAE tubulin was incubated on ice for 5 min in BRB80 buffer with 1 mM DTT and 1 mM GMPCPP (guanosine-5'-(β,γ)-methylene)triphosphate; Jena Bioscience) and clarified by centrifugation at 90,000 rpm in a rotor (TLA100.4; Beckman Coulter) for 5 min at 4°C. Clarified tubulin was polymerized at 37°C for 60 min and centrifuged at 90,000 rpm for 5 min at 37°C. The MT pellet was resuspended in pre-warmed BRB80 buffer with 25 mM taxol and 1 mM DTT (0.8 volume of starting polymerization volume).

**MT-stimulated ATPase activity.** MT-stimulated ATPase activity was determined by measuring phosphate release at 25°C with the P/COLOR reagent (Innova Biosciences) in a modified malachite green assay. 50–150 nM KIF17M-490 alone or with KIF17-tail (equimolar with KIF17-M) and EB1 (2x molar ratio) was incubated with MTs in BRB80, pH 6.9, containing 75 mM KCl and 1 mM DTT. Phosphate release was plotted against MT concentration, and data were fit to a Michaelis–Menton equation using Prism (GraphPad Software) to determine $k_{cat}$ and $K_m$. No ATP hydrolysis was detected when MTs, EB1, or KIF17-tail were assayed in the absence of KIF17M-490.

The phasor transformation

Globals software (Laboratory for Fluorescence Dynamics, University of Illinois at Urbana-Champaign, Urbana, IL) was used to analyze fluorescence data. Box–whisker plots show minimum, 25th percentile, median, 75th percentile, maximum, and mean FRET values. Statistical significance was determined using a two-tailed Student’s $t$ test. Confidence limits for FRET analysis (Caiolfa et al., 2007; Barreiro et al., 2008). Two phasor subsets are shown in the plots, and corresponding pixels are mapped in the FLM images: pixels highlighted by the red mask have FRET efficiency $<7\%$ and represent KIF17 in the extended or active form, and pixels highlighted by the green mask have FRET efficiency between 7–21% and represent KIF17 in the compact, inactive conformation.

FRET efficiency is obtained by localizing the positions of phasor clusters in the phasor plot. The position of the measured point on the trajectory gives the FRET efficiency. Because the fluorescence decay in each pixel of the image gives a point in the phasor plot, the location of the measured point is the intensity-weighted mean of FRET, non-FRET, and autofluorescence components. Thus, for each pixel in which the contribution of the non-FRET component is high (i.e., autofluorescence), points on the phasor plot will approach the green line (representing 0% FRET efficiency). Conversely, in pixels in which the contribution of the FRET component is high, points on the phasor plot approach the red line (representing 50% FRET efficiency). The curved trajectory on the phasor plot represents realizations of all possible phasors quenched with different efficiencies.

**References**


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Figure S1. **Characterization of KIF17 for FLIM.** (A) KIF17 activity is regulated by an autoinhibition mechanism. MT-stimulated ATPase activity of KIF17-M490 alone, in the presence of EB1 or in presence of KIF17 tail domain. Error bars show SDs. (B) FLIM measurements and phasor analysis. Representative FRET/FLIM analysis of controls using the phasor plot. Sine (s) and cosine (g) transforms of the lifetime data measured in the frequency mode generate the coordinate system presented in the phasor. FLIM-transformed decay data at each pixel are shown in contour plots for cells transfected with donor only (EmGFP) or with a control linker construct (mCh-16aa-EmGFP). Autofluorescence in untransfected cells is represented by the background. The green line represents 0% FRET efficiency, and the red line marks 50% FRET efficiency. Lines drawn in green (for donor only), black (for the linker), and red are trajectories joining the mean values of the phasor distributions in cells at different contributions of autofluorescence (0–100%). The curved trajectory corresponds to FRET efficiency (from 0 to 100%). This trajectory is computed using the phasor of the donor in the absence of FRET (donor only) and the phasor of the background, which were determined independently, and applying the classic definition of FRET efficiency = \(1 - (\varphi_{\text{donor-in}})/\varphi_{\text{donor}}\), to determine the phasor corresponding to the quenched donor. The position of the phasor of a given sample along the trajectory determines the amount of quenching and, therefore, the FRET efficiency. (C) Mean FRET efficiency of the linker derived from the phasor distributions acquired in MDCK cells. (D) MDCK cells expressing mCh-KIF17-EmGFP were permeabilized with 0.01% saponin in the presence of 1 mM AMP-PNP for 10 min. Fluorescence images (insets) and the positions of KIF17 in an extended, active conformation (red mask on image) determined from measured lifetimes before and after treatment with AMP-PNP. Bars, 20 μm. (E and F) Box-whisker plots showing the distribution of FRET (left) and the fraction of extended (right) KIF17 in control and AMP-PNP-treated MDCK cells. Box-whisker plots show minimum, 25th percentile, median, 75th percentile, maximum, and mean FRET values. Data were obtained from three experiments ± SEM.
Figure S2. Selective depolymerization of stable MTs with OA results in loss of active KIF17. (A) Immunostaining of tyrosinated, detyrosinated (Glu), and acetylated tubulin in untreated MDCK cells or cells treated 90 min with 100 nM okadaic acid (OA). Bar, 20 µm. (B) Box-whisker plots showing the distribution of FRET$_{eff}$ (left) and the fraction of extended (right) KIF17 in control and OA-treated cells. Data were obtained from three independent experiments ± SEM. Box-whisker plots show minimum, 25th percentile, median, 75th percentile, maximum, and mean FRET values.
Figure S3. **KIF17 localizes on dynamic and stable MTs in MDCK cells.** (A) MDCK cells expressing the hinge mutant GFP-KIF17^G754E^ were fixed in methanol 2 h after cDNA microinjection (Inj). In untreated cells (left column), GFP-KIF17^G754E^ localizes at the plus ends of MTs in cell protrusions (arrows). This localization is lost (arrowheads) in cells treated for 1 h with 10 µM taxol before injection (middle column) or 2 h after injection (right column). Bars, 20 µm.

(B) Cells expressing KIF17-EmGFP were permeabilized with 0.01% saponin in the presence of 1 mM AMP-PNP and then fixed in methanol for immunofluorescence analysis. Cells were immunostained for tyrosinated (Tyr), acetylated (Ace), and detyrosinated (Glu) tubulin. Boxed regions in grayscale images are enlarged and shown in color overlays at the right. In color overlays, the GFP image was shifted to emphasize the similar patterns of MTs and KIF17-EmGFP. Bars, 10 µm.