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Report

Costal2 Functions as a Kinesin-like Protein in the Hedgehog Signal Transduction Pathway

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Summary

The Hedgehog (Hh) signaling pathway initiates an evolutionarily conserved developmental program required for the proper patterning of many tissues [1]. Although Costal2 (Cos2) is a requisite component of the Hh pathway, its mechanistic role is not well understood. Because of its primary sequence, Cos2 was initially predicted to function as a kinesin-like protein [2]. However, evidence showing that Cos2 function might require kinesin-like properties has been lacking [2–6]. Thus, the prevailing dogma in the field is that Cos2 functions solely as a scaffolding protein [7, 8]. Here, we show that Cos2 motility is required for its biological function and that this motility may be Hh regulated. We show that Cos2 motility requires an active motor domain, ATP, and microtubules. Additionally, Cos2 recruits and transports other components of the Hh signaling pathway, including the transcription factor Cubitus interruptus (Ci). *Drosophila* expressing *cos2* mutations that encode proteins that lack motility are attenuated in their ability to regulate Ci activity and exhibit phenotypes consistent with attenuated Cos2 function [9]. Combined, these results demonstrate that Cos2 motility plays an important role in its function, regulating the amounts and activity of Ci that ultimately interpret the level of Hh to which cells are exposed.

Results and Discussion

Consistent with its role as a scaffolding protein [3–6], Costal2 (Cos2) associates with the transcription factor Ci [10, 11], the transmembrane protein Smoothened (Smo) [12–15], and a number of protein kinases [3, 4, 16, 17] that regulate the Hh pathway. Although Cos2 also associates with membrane vesicles [18] and microtubules (MTs) [3] in a manner regulated by Hh, its functional relationship with these subcellular structures is largely unknown. To explore Cos2's binding with both MTs and vesicular membranes, we expressed a *GFP*-tagged wild-type (WT) *cos2* construct (*cos2-GFP*), similar to one capable of rescuing *cos2* mutant *Drosophila* [9], and determined its subcellular location in the Hh-responsive *Drosophila* S2 cultured cell line [19, 20] (Figure 1A). The Cos2-GFP protein enriched in various-sized puncta throughout the cytoplasm (Figure 1A). The number and relative size of these puncta appeared to be variable within the population of cells, as did the amount of Cos2 that localized in a more diffuse manner (Table S1 available online). Hh attenuated the localization of Cos2 to these distinct puncta, as evidenced by the fact that Cos2-GFP localization appeared relatively more diffuse in S2 cells exposed to Hh (Figure 1A′), but Hh had little effect on Cos2-GFP levels in cells (see Figure S1G). Similar results were obtained when *smo* fused to *GFP* (*smo-GFP)* was coexpressed with *cos2-RFP* (Figure 1B′ compared to Figure 1B and Table S1) because high levels of Smo are known to activate Hh signaling [21]. The number of cells exhibiting diffuse Cos2-RFP localization, as compared to the number of cells with Cos2-RFP in puncta, increased as the amount of *smo-GFP* transfected was increased.

Endogenous Cos2 also localized to different-sized punctate structures in S2 cells (Figure 1C and Figure S1A) and in *Drosophila* embryos [22]. Furthermore, this punctate localization of Cos2 is significantly altered in cells exposed to Hh, which exhibit a more diffuse localization (Figure 1C′ compared to Figure 1C, Figure S1A, and Table S1). These puncta indeed represent localization of endogenous Cos2 because they are largely absent in S2 cells treated with Cos2-specific dsRNA (Figure S1D). Taken together, our observations are consistent with Cos2-GFP localizing in a manner similar to endogenous Cos2, both in the presence and absence of Hh.

Cos2 comigrates with vesicular markers, suggesting that some fraction of the Cos2 puncta we observed was localized to discrete membrane vesicles [18]. To begin to identify to what types of vesicles Cos2 localizes, we expressed *cos2-GFP* and probed for a series of endogenous vesicular markers (Figure 1D, Figure S2, and Table S2). We obtained significant overlap of Cos2-GFP puncta with markers of early endosomes (~30%) (Figure 1D and Figure S2), recycling endosomes (Figure S2B), and late endosomes (Figures S2C and S2D). Thus, a subset of the Cos2 puncta observed here probably represents endosomal vesicles.

To identify what factors might regulate Cos2 localization to these puncta, we knocked down Smo and Fu expression by RNAi and examined the ability of Cos2 to translocate from a membrane-enriched fraction to a cytoplasmic fraction (Figure S1B). Consistent with Smo and Fu being required for Hh signaling [23–25], their knockdown attenuated the ability of Cos2 to translocate from the membrane-enriched fraction to a cytoplasm-enriched fraction in response to Hh. Hh normally induces the Fu-dependent phosphorylation of Cos2 [3, 26]. However, overexpression of both *fu* and *cos2* leads to the phosphorylation of Cos2 [26], suggesting that Fu may be constitutively active when overexpressed with Cos2 (see also Figure S1F). Consistent with this latter suggestion, coexpression of *fu* with *cos2-GFP* also affected the resulting protein's subcellular distribution in a dose-dependent manner, with much of Cos2-GFP appearing more diffuse in the presence of high

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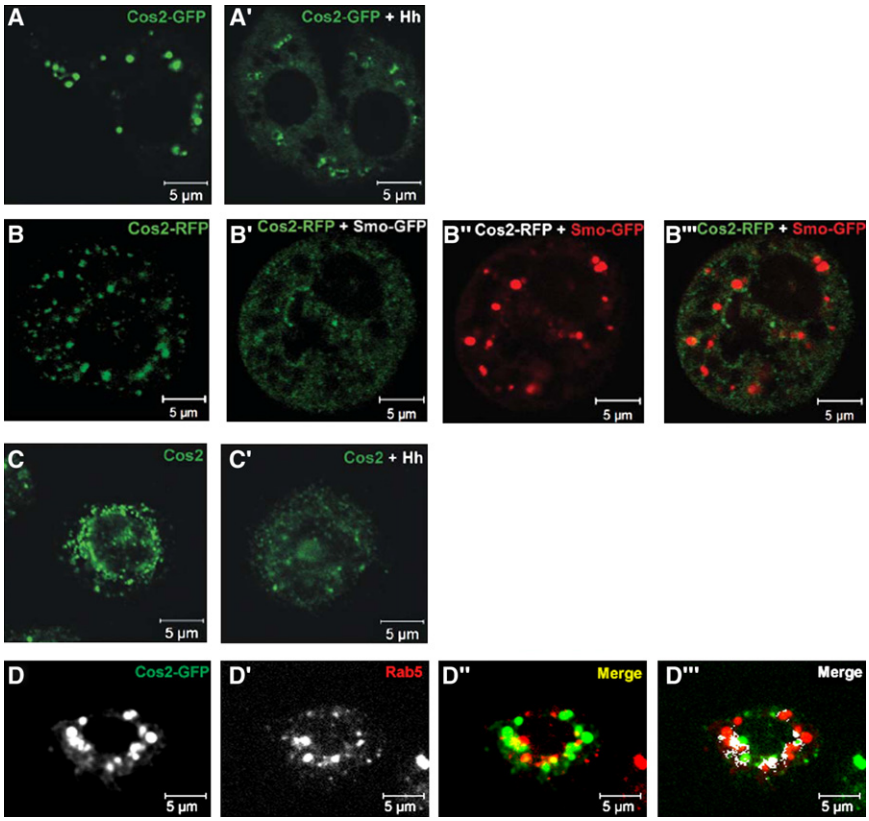

Figure 1. Hh Regulates the Subcellular Localization of Cos2

(A and A′) Images of live S2 cells expressing *cos2-GFP* with (+) or without *hh*.
(B–B‴) Images of live S2 cells expressing *cos2-RFP* only (B), pseudo-colored green, or coexpressing *smo-GFP* (B′–B‴), pseudo-colored red.
(C and C′) Indirect immunofluorescent staining of endogenous Cos2 in S2 cells, with (+) or without *hh*.
(D–D‴) Cos2-GFP puncta partially colocalize with Rab5. In (D‴), the image shows Cos2 and Rab5 colocalization with areas of overlap highlighted in white. Scale bars in all panels represent 5 μm.

levels of exogenous Fu (Figure S1E and data not shown). Interestingly, Fu is also able to relieve Cos2's inhibition of Ci activity, correlating Cos2's punctate localization with its ability to attenuate Ci activity (Figure S1F). These results suggested a positive role for Smo and Fu in Hh-dependent Cos2 relocalization.

Many of the Cos2-GFP puncta appeared to move rapidly throughout the cytoplasm of S2 cells (Figure 2A, Movie S1, and Movie S2). The Cos2-enriched puncta exhibited a velocity inversely related to their size, with smaller puncta moving more quickly than larger puncta (Figure S3c, Movie S1, and Movie S2). For ease of comparison, we divided the different-sized puncta into three groups, designated large, medium, or small (Figure S3A), and compared their velocities (Figure S3C and Table S3). The average velocity of small Cos2-GFP puncta was 62 nm/s, which was within the range reported for kinesin-like proteins (KLPs) [27–30], but less than our positive control, Klp10A [31, 32] (Figure 2B and Table S3). Many Cos2-GFP puncta were quite dynamic in nature, with small puncta appearing to fuse with and even bud off from the larger puncta (Figure S4A and Movie S16). Photobleaching experiments verified the dynamic nature of Cos2-GFP populating the larger puncta (Figure S4B and Movie S17), which quickly recovered from the photobleaching procedure.

To confirm that Cos2-GFP movement was an active process dependent on its kinesin-like activity, we compared the movement of Cos2-GFP to Cos2ΔMotor-GFP (Figure 2C; also see Movie S1 compared to Movie S4), a mutant Cos2 fusion protein that lacks its putative motor domain and would be predicted to be immotile. A similar motorless GFP fusion protein has been shown to function as a dominant negative inhibitor of WT Cos2 in vivo [9], presumably through association with endogenous WT Cos2. Although some minor movement of Cos2ΔMotor-GFP puncta was observed, Cos2ΔMotor-GFP mobility was significantly reduced relative to that of Cos2-GFP (compare Figure 2A′ with Figure 2C′, as well as Table S3). Immobile Cos2ΔMotor-GFP puncta were also more numerous and larger than those observed with WT Cos2-GFP (Figure 2c). As the cargos of KLPs typically associate with both anterograde motors and retrograde motors, residual movement of a small subset of Cos2ΔMotor-GFP puncta may be due to transport by another motor protein [33].

At its most basic level, KLP motor activity requires ATP and intact MTs [34, 35]. Therefore, we examined the ability of Cos2 to move in the absence of either ATP or an intact MT network (Figure 3). S2 cells that were depleted of ATP [36] still contained Cos2 puncta, but these puncta were almost completely immobile (Figures 3A and 3A′, Table S3, and Movie S5). However, upon restoration of ATP levels, Cos2-GFP puncta motility was quickly reestablished (Figures 3B and 3B′ and Movie S6). To then test whether these Cos2 puncta colocalize with MTs, we expressed *cos2-RFP* in cells stably expressing *GFP-α-tubulin*. Whereas the bulk of RFP alone was predominantly nuclear (data not shown), the majority of punctate Cos2-RFP appeared to colocalize with the MT network (Figures 3C and S5B). We next used nocodazole to disrupt the MT network of individual S2 cells expressing *cos2-GFP* and examined the localization of Cos2-GFP to discrete MT-associated puncta with live imaging (Figures 3D and S5A). The loss of punctate Cos2-GFP in a nocodazole-treated cell correlated with the destabilization of the MT network, with loss of peripheral MTs and peripheral puncta preceding loss of more central MTs and puncta. These results show that Cos2 colocalizes with MTs and that Cos2's enrichment in punctate structures depends on an intact MT network. Because Hh is known to disrupt the physical association between Cos2 and MTs [2, 3], Hh might control Cos2's ability to form motile puncta by regulating its MT association. We note, however, that despite its lack of a motor domain, Cos2ΔMotor-GFP is still able to form puncta (Figure 2C). We hypothesized that either Cos2ΔMotor-GFP is able to bind MTs through a domain distinct from its motor domain, which normally harbors a MT-binding motif [37], or that Cos2ΔMotor-GFP's ability to form puncta is due to dimerization with endogenous WT Cos2. Consistent with this latter suggestion, immunoprecipitation of Cos2ΔMotor-GFP was able to coprecipitate full-length WT Cos2 (Figure S3D).

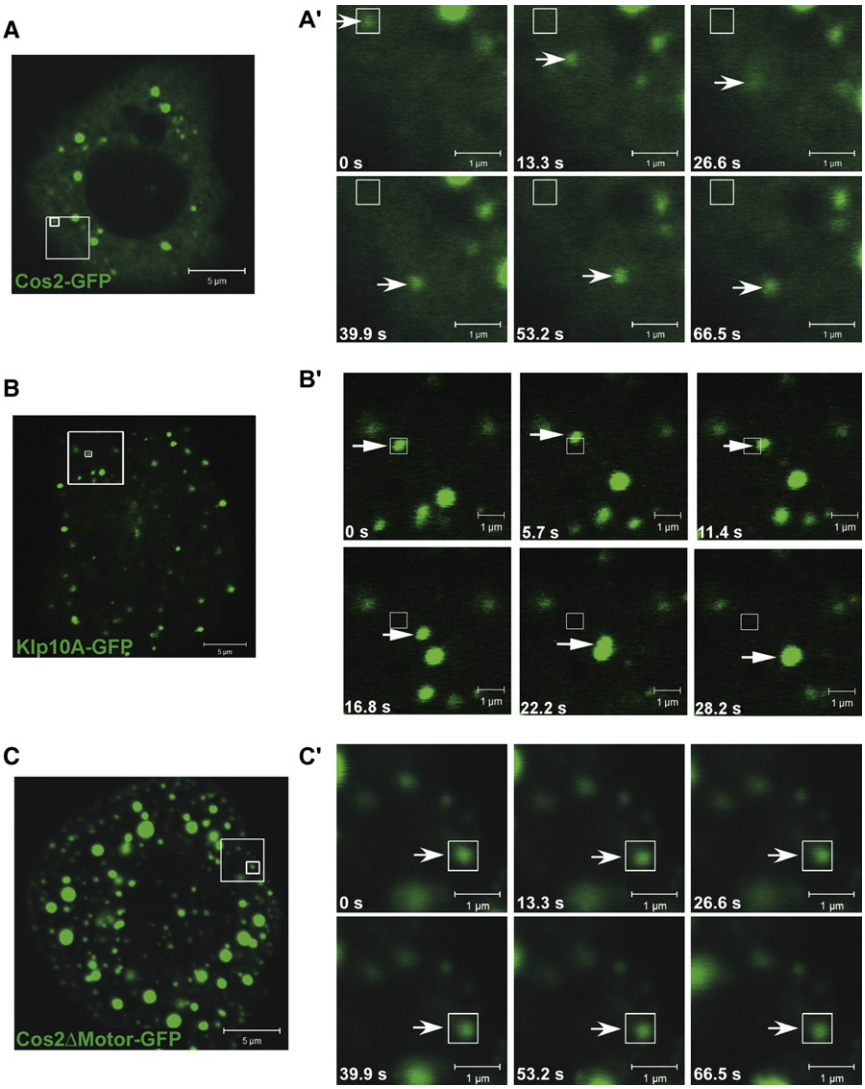

Figure 2. Cos2 Puncta Are Motile

S2 cells expressing *cos2-GFP* (A); *klp10A-GFP* (B), a known kinesin family member; or *cos2Δ-Motor-GFP* (C), a GFP-fused truncated form of Cos2 lacking its putative motor domain. The large box in each panel indicates the enlarged area depicted in the corresponding time series in (A′)–(C′). The smaller box indicates the origin of a single punctum that is tracked over time with arrows in (A′)–(C′); the time-lapse images therein show a Cos2-GFP punctum (A′), a Klp10A-GFP punctum (B′), and a Cos2ΔMotor-GFP punctum (C′) that remains essentially immobile over the same time period as in (A′). Scale bars represent 5 μm in panels (A)–(C) and 1 μm in (A′)–(C′).

To more directly establish that Cos2 puncta move along MTs, we analyzed Cos2-RFP movement in cells expressing *GFP-α-tubulin* and observed many smaller Cos2-RFP puncta that appeared to track along MTs (Figure S5B and Movie S7) [38]. To visualize longer and more defined MTs, we induced S2 cells expressing *cos2-GFP* and *mCherry-tubulin* to form long, thin, MT-enriched cellular extensions by treating them with the actin-disrupting agent cytochalasin-D (Figure 3E) [39]. Under these conditions, Cos2-GFP puncta also appear to move along these longer MT extensions (Movie S8 and Movie S9). Approximately 80% of the Cos2-GFP puncta that localized to these MT-enriched cellular extensions exhibited significant mobility (Table S4).

Molecular motors utilize ATP hydrolysis to generate force, which is then translated into movement [40]. A putative Cos2 ATPase-deficient mutant, Cos2-S182N, functions as a dominant negative inhibitor of endogenous Cos2 in vivo [9], suggesting that Cos2 requires ATPase activity for function. We expressed *GFP*-tagged *cos2-S182N* in cells to determine the contribution of ATPase activity to Cos2's ability to move along MTs. Consistent with the prediction that this mutation should lack motor activity, we found that although Cos2-S182N-GFP was able to enrich in puncta, these puncta did not exhibit any significant motility (Figure 3F, Table S3, and Movie S10). Thus, in the absence of a functional ATPase domain, the mobility of Cos2-GFP-enriched puncta is significantly attenuated, exhibiting motility comparable to that observed in ATP-depleted S2 cells (Figure S3B and Table S3). In general, the localization of Cos2-S182N-GFP appeared more diffuse than that observed with WT Cos2-GFP (data not shown). This increased degree of diffuse localization of Cos2 is consistent with a decreased affinity for MTs. Interestingly, mutations in conserved nucleotide-binding motifs in other KLPs can also affect their MT affinity [41, 42]. Taken together with the phenotype of this *cos2-S182N-GFP* mutant in *Drosophila* [9], these results, along with those observed with Cos2ΔMotor-GFP, suggest that Cos2 ATP-dependent motility is crucial for its activity.

If Cos2 is able to move along the MT network, it is likely to transport its various binding partners throughout the cell. To test this hypothesis, we coexpressed WT *cos2* with either the carboxyl-terminal domain of Fu tagged with GFP (*GFP-fu-tail*) or a region of Ci tagged with GFP (*GFP-ci-CORD*) because both of these regions of Fu and Ci associate with Cos2 [4, 10, 43] (Figures 4B and 4D and Movie S11 and Movie S13). When expressed on their own, GFP-Fu-tail and GFP-Ci-CORD localized diffusely throughout the cytoplasm and nucleus, whereas GFP-Ci-CORD was predominantly nuclear [44] (Figures 4A and 4C). However, when either *GFP-fu-tail* or *GFP-ci-CORD* was coexpressed with WT untagged *cos2,* each relocalized to discrete motile puncta (Figures 4B and 4D) that exhibited a similar velocity to that observed for Cos2-GFP alone (data not shown). Moreover, a high degree of colocalization and comovement of Cos2-RFP with Fu-tail-GFP and with Ci-CORD-GFP was also observed (Figures S6A and S6B, Table S3, and Movie S12 and Movie S14). The velocity of these colabeled puncta was similar to that of Cos2-GFP puncta alone (Figure S3B and Table S3). These results suggest that Cos2 has the ability to recruit Fu and Ci to discrete puncta and transport them through the cytoplasm. Consistent with this suggestion, Fu, Cos2, and Ci appear to colocalize in various-sized puncta when all three are coexpressed in S2 cells (Figure S6D).

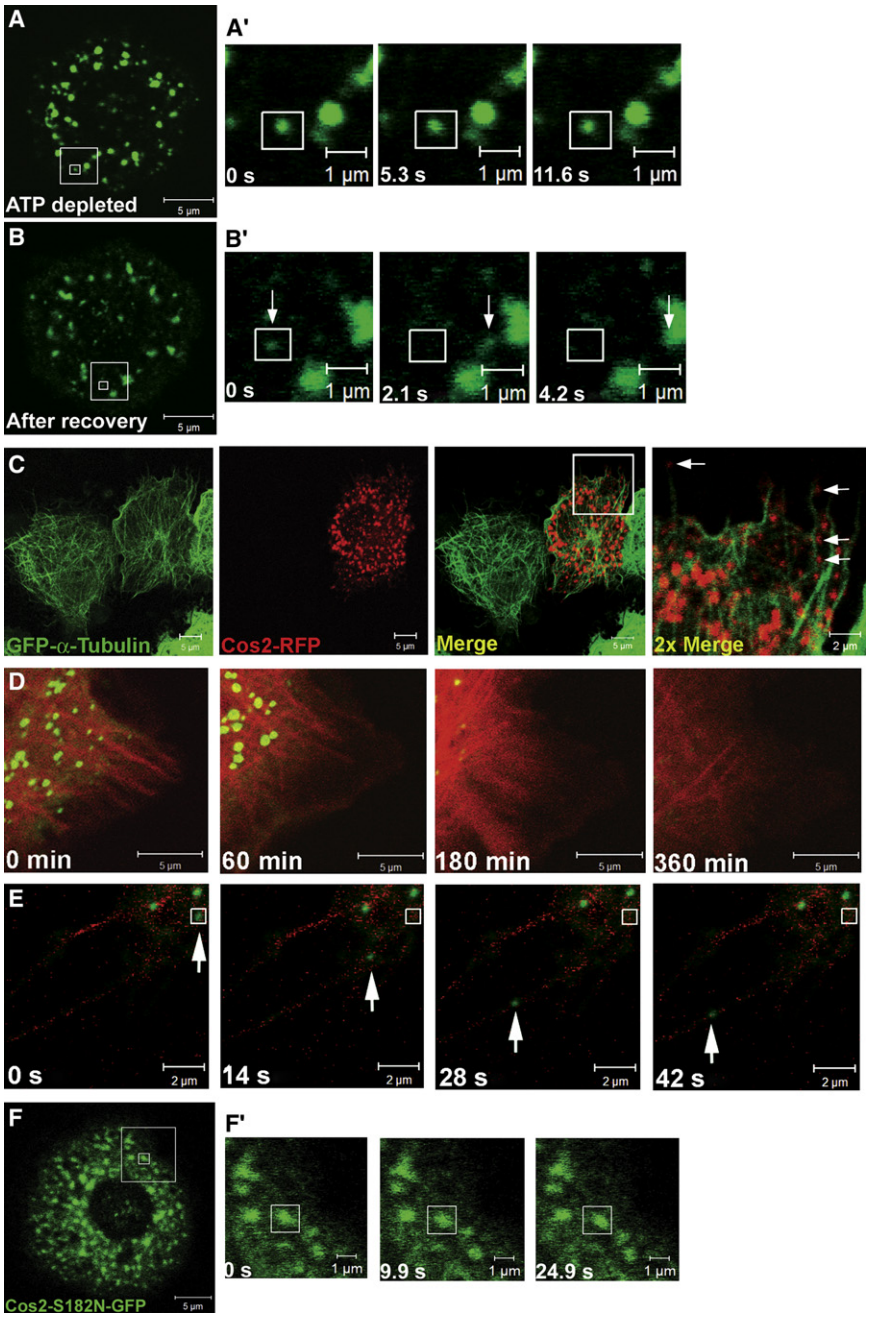

Figure 3. Cos2 Motility Requires Microtubules and ATPase Activity

The large box in (A) and (B) indicates the enlarged area depicted in the corresponding time series (A′ and B′). The smaller boxes indicate the origin of a single punctum that is tracked over time.

(A–A′) S2 cells expressing *cos2-GFP* were ATP depleted by treatment with 5 mM sodium azide and 1 mM 2-deoxyglucose. Time-lapse images were taken 30 min after ATP depletion.

(B–B′) Cos2 movement is recovered after ATP levels are restored, verifying the reversibility of the ATP depletion. The same cell as in (A) was re-imaged after 30 min of recovery in fresh media. The movement of a single punctum (indicated by arrows) is tracked over time.

(C) Cos2 appears to localize along MT tracks. *cos2-RFP* was expressed in an S2 cell line stably expressing *GFP-α-tubulin* and imaged in live cells. The large box in the merge panel indicates the enlarged area depicted in the 2× merge panel. Individual Cos2-RFP puncta aligning with MTs are marked by arrows in the rightmost panel.

(D) Images of a single live S2 cell cotransfected with plasmids expressing *cos2-GFP* and *mCherry-tubulin* and then treated with nocodazole for 6 hr.

(E) Images of live S2 cells expressing *cos2-GFP* and *mCherry-tubulin* that were treated with 5 μM cytochalasin for 18 hr. Cytochalasin disrupts the actin cytoskeleton, allowing the formation of long MT-enriched projections, along which Cos2-GFP puncta appear to colocalize and move.

(F–F′) Images of live S2 cells expressing *cos2-S182N-GFP*. (F) Cos2-S182N-GFP shows a highly punctate staining pattern in a subset of cells, similar to WT Cos2-GFP. The large box in panel (F) indicates the enlarged area depicted in the corresponding time series (F′), and the smaller box indicates the origin of a single punctum tracked over time. Cos2-S182N-GFP puncta tracked over time in (F′) do not exhibit significant motility (see also Figure S3B). Scale bars represent 5 μm in (A), (B), (D), and (F), 2 μm in (C) and (E), and 1 μm in (A′), (B′), and (F′).

Because a fraction of Cos2 also associates with Smo [12], we examined the ability of this Smo/Cos2 complex to form and move together throughout the cell. As shown above (Figure 1B), high levels of *smo* result in a more diffuse distribution of Cos2-GFP, consistent with high levels of Smo constitutively activating Hh signaling [21]. However, when *cos2-RFP* was coexpressed with lower levels of *smo-GFP* in S2 cells, we observed a significant degree of overlap between the two proteins (Figure 4E). We also observed that many of the puncta enriched for both Cos2-RFP and Smo-GFP appeared to move together throughout the cell (Figure 4E′ and Movie S15). The Smo-GFP/Cos2-RFP puncta had a velocity approximately three times that of Cos2-GFP alone or that of puncta containing Cos2-GFP with Ci-CORD or Fu-tail (Table S3). The reasons for this difference in apparent velocity are currently unknown, but the finding is consistent with the puncta containing the Smo/Cos2 complex being distinct from the major pool of Cos2 puncta [45].

Our results demonstrate that Cos2 displays many of the hallmarks of a KLP, exhibiting MT-dependent motility that is dependent on a functional motor domain and on ATP. We show that Hh regulates the motility of Cos2 through directly regulating Cos2 movement and/or through regulating Cos2's affinity with MTs, which may require Fu activity. KLPs are known to transport specific cargos, which are distinct for the various family members. Our results suggest that one important cargo for Cos2 is the transcription factor Ci, which ultimately determines all Hh readouts [46]. Although we cannot currently rule out the possibility that the motility we observe is indirect, we suggest that Cos2 motility is an intrinsic property of the protein. Interestingly, precedence for KLP motility regulating the activity of a transcription factor was recently provided in a study linking TGF-β signaling to kinesin-1 function [47]. The activation and nuclear accumulation of the transcription factor SMAD2

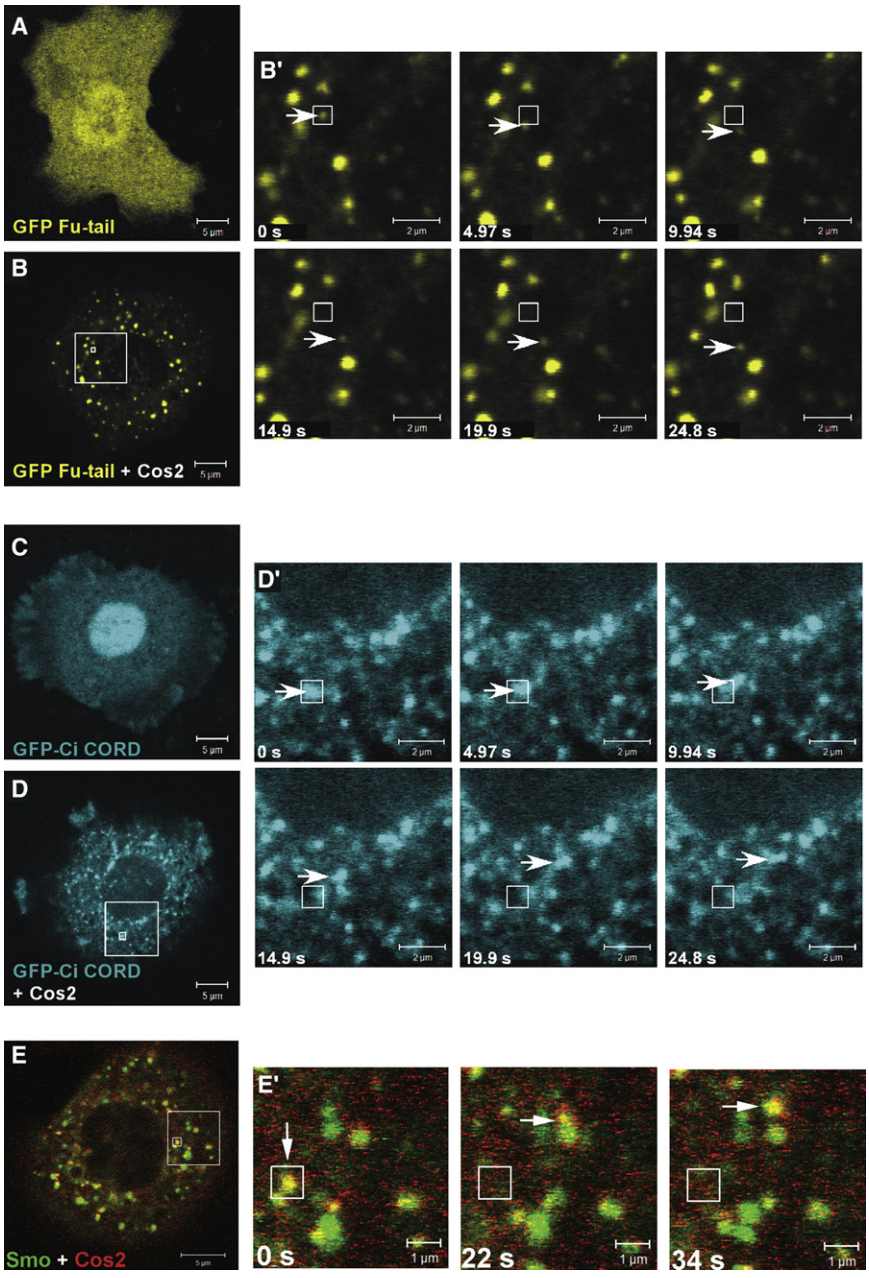

Figure 4. Cos2 Recruits and Moves Other Components of the Hh Signaling Pathway

GFP-fu-tail (A and B) and *GFP-ci-CORD* (C and D) were expressed in S2 cells in the presence or absence of exogenous WT *cos2* and then analyzed by live imaging as described in the text. Images in (E) of live S2 cells expressing *cos2-RFP* and *smo-GFP* show that a subset of puncta colocalizes and is motile (E′). Corresponding single fluorescence images of Smo-GFP and Cos2-RFP are shown in Figure S6C. The large box in (B), (D), and (E) indicates the enlarged area depicted in the corresponding time series (B′), (D′), and (E′). The smaller boxes indicate the origin of a single particle (marked by arrow) tracked over time. Scale bars represent 5 μm in (A)–(E), 2 μm in (B′) and (D′), and 1 μm in (E′).

was shown to be dependent upon kinesin-1 trafficking, with the loss of kinesin-1-dependent SMAD2 motility ultimately resulting in attenuated TGF-β signaling [47]. Similarly, Hh appears to regulate the Cos2-dependent movement of many of its signaling components, including Ci.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, six figures, four tables, and seventeen movies and can be found with this article online at http://www.current-biology.com/cgi/content/full/18/16/1215/DC1/.

Acknowledgments

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