

Kinesin Autoinhibition Requires Elbow Phosphorylation

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eLife Assessment

In this **important** manuscript, the authors investigate the phospho-regulation of the *C. elegans* kinesin-2 motor protein OSM-3, revealing that the kinase, NEKL-3, phosphorylates a serine/threonine patch at the hinge region of the motor to mediate autoinhibition until it reaches the ciliary middle segment. The findings are supported by robust genetic data, in vivo imaging, and motility assays with wild-type and mutant motors, although the methods section lacks detailed protocols for NEKL-3 assays and in silico analyses. Overall, the study provides a **solid** contribution to understanding the regulation of OSM-3 kinesin activity.

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Abstract

Kinesin motor proteins facilitate microtubule-based transport by converting chemical energy into mechanic forces, but this activity is autoinhibited until cargo is loaded. Regulatory mechanisms underlying this autoinhibitory conformation are not well understood. Here, we show that a NEver in mitosis Kinase NEKL-3 directly phosphorylates a flexible elbow region between two coiled coil domains connecting the motor head and tail of an intraflagellar transport kinesin, OSM-3. The phosphor dead (PD) mutation, but not phosphor mimic (PM) mutation, induce constitutive motility of OSM-3 in vitro. Using knock-in animals, we discovered that both PD and PM mutations shorten the *C. elegans* sensory cilia. The hyperactive OSM-3PD fails to enter cilia and abnormally accumulates in neurites, mimicking another hyperactive mutation, OSM-3G444E. Conversely, OSM-3PM enters cilia but moves at a reduced speed, indicating an inhibitory role of elbow phosphorylation in kinesin motility. These findings highlight the crucial role of elbow phosphorylation in regulating kinesin autoinhibition.

Introduction

Kinesin motor proteins, integral to intracellular transport along microtubules, are tightly regulated for precise cargo delivery within cells (Burute and Kapitein, 2019 [DOI](#); Christensen and Reck-Peterson, 2022 [DOI](#); Hirokawa et al., 2009 [DOI](#); Ou and Scholey, 2022 [DOI](#)). Dysregulation of kinesin activity is linked to neurodegenerative disorders like Alzheimer's and Parkinson's diseases (Brady and Morfini, 2017 [DOI](#); Sleight et al., 2019 [DOI](#)). Understanding these regulatory mechanisms is vital for elucidating disease mechanisms and developing potential therapeutic strategies. Mechanisms controlling kinesin activation involve protein interactions, post-translational modifications, and cargo binding (Cason and Holzbaur, 2022 [DOI](#); Verhey and Hammond, 2009 [DOI](#)). Protein phosphorylation is recognized for its regulatory role in a variety of proteins, yet its impacts on kinesin motility are not fully understood (Banerjee et al., 2021 [DOI](#); DeBerg et al., 2013 [DOI](#); Espeut et al., 2008 [DOI](#); Liang et al., 2014 [DOI](#); Sato-Yoshitake et al., 1992 [DOI](#); Schäfer et al., 2008 [DOI](#)).

Cilia serve as a unique model for studying how microtubule-based motor proteins are regulated in specific cellular regions (Anvarian et al., 2019 [DOI](#); Nachury and Mick, 2019 [DOI](#)). The formation of cilia relies on bidirectional intraflagellar transport (IFT) along microtubules within the axoneme (Ishikawa and Marshall, 2011 [DOI](#); Klena and Pigino, 2022 [DOI](#); Taschner and Lorentzen, 2016 [DOI](#)). At the ciliary base, kinesin-2 family proteins undergo conformational changes to transport IFT-particles loaded with ciliary precursors to the axonemal tip (Ou and Scholey, 2022 [DOI](#); Taschner and Lorentzen, 2016 [DOI](#)). After unloading cargo, kinesin-2 is deactivated, while dynein-2 is activated to recycle the anterograde IFT machinery (Ou and Scholey, 2022 [DOI](#); Prevo et al., 2017 [DOI](#); Taschner and Lorentzen, 2016 [DOI](#)). In *Caenorhabditis elegans*, kinesin-II and OSM-3 collaborate to construct sensory cilia in chemosensory neurons (Ou et al., 2005 [DOI](#); Ou and Scholey, 2022 [DOI](#); Prevo et al., 2015 [DOI](#)). Initially, kinesin-II transports IFT-particles, building the middle ciliary segments, while OSM-3 is inactive and transported by kinesin-II. Later, kinesin-II transfers IFT particles to OSM-3, activating it to convey cargo molecules and assemble the distal axoneme. The molecular mechanisms that govern the activation and inactivation of kinesin-II and dynein-2 along the cilia are still unclear. Additionally, the regulation of OSM-3's regional motility presents an even greater mystery: Deleting OSM-3 only disrupts the distal axoneme without affecting the ciliary middle region. How does OSM-3 become more enriched in the distal domain than the middle segment?

The “elbow” of a kinesin refers to a flexible region situated between two coiled-coil domains, linking the motor head and tail of the kinesin protein (Tan et al., 2023 [DOI](#); Weijman et al., 2022 [DOI](#)). This junction imparts necessary flexibility and facilitates interactions between the globular motor head and the rigid stalk-like tail (Tan et al., 2023 [DOI](#); Verhey and Hammond, 2009 [DOI](#); Weijman et al., 2022 [DOI](#)). The elbow region is thus considered to serve as a pivotal regulatory center, governing the motor's activity and facilitating precise spatiotemporal control of intracellular transport processes (Tan et al., 2023 [DOI](#); Weijman et al., 2022 [DOI](#)). However, the post-translational modification of the kinesin elbow and its physiological consequences have not been elucidated. In this study, we demonstrate that the NEKL-3 kinase directly phosphorylates the elbow of OSM-3 kinesin. Our in vitro and in vivo findings suggest that elbow phosphorylation exerts an inhibitory effect on OSM-3 motility. We show that elbow phosphorylation inhibits OSM-3 motility in the soma and dendrites of sensory neurons, while its dephosphorylation is necessary for activating OSM-3 in sensory cilia.

Results

NEKL-3 phosphorylates the OSM-3 kinesin's elbow

We found that NEKL-3, a member of the MIMA kinase family, directly phosphorylates OSM-3. Our mass spectrometric analysis identified phosphorylation sites at residues 487-490, corresponding to the amino acid sequence “YSTT” located in OSM-3's tail region (**Fig. 1A-B**). These four residues are situated at the end of the hinge2 region, linking coiled-coil1 and coiled-coil2 (**Fig. 1A, C**). In kinesin-1, this inter-coiled-coil junction is known as the “elbow,” essential for adopting a compact conformation for autoinhibition (Tan et al., 2023; Weijman et al., 2022). Accordingly, we have designated the region comprising amino acids 487-490 as the “elbow” of OSM-3. Although the elbow region is prevalent throughout the kinesin superfamily, the molecular regulation of the elbow conformation remains to be illustrated.

Phosphorylation at the elbow inhibits the motility of the OSM-3 kinesin in *C. elegans*

To investigate the in vivo effects of phosphorylation and dephosphorylation at the “elbow” region of OSM-3, we utilized genome-editing techniques to generate knock-in worms harboring phospho-dead (PD) and phospho-mimic (PM) mutations. Specifically, we replaced the amino acids 487-490 YSTT with FAAA for the PD mutation and DDEE for the PM mutation in the *C. elegans* OSM-3::GFP genome (**Fig. 1D-E**). The wild-type (WT) OSM-3::GFP facilitates anterograde IFT to construct the distal ciliary segments of sensory neurons, and OSM-3::GFP fluorescence localizes along the ciliary distal segment. However, OSM-3PD::GFP was absent from cilia and excluded from cell bodies, instead forming bright puncta around the axons of sensory neurons (**Fig. 2A-B**). By introducing an IFT marker IFT70/DYF-1::mScarlet into OSM-3PD worms, we revealed a marked reduction in ciliary length compared to WT animals, consistent with the absence of OSM-3 kinesin within the cilia (**Fig. 2B-C**). The shortened cilia length and the formation of abnormal puncta at the neurite tip resemble the phenotype observed in our previously characterized OSM-3G444E mutation, which disrupts autoinhibition and leads to hyperactivation of OSM-3 (Xie et al., 2024).

Conversely, OSM-3PM::GFP fluorescence did not form puncta but localized within the sensory cilia, albeit with shortened cilia and reduced IFT speed, indicating impaired OSM-3 motility (**Fig. 2B-D**, Fig. S1). These findings suggest that NEKL-3-mediated phosphorylation of the OSM-3 elbow may exert inhibitory effects on OSM-3 motor activity. Dephosphorylation of the elbow relieves autoinhibition, resulting in a constitutively active form of OSM-3 unable to be transported to the cilium. Conversely, constitutive phosphorylation of the elbow may maintain its autoinhibition, enabling transport to the cilium but causing slow IFT.

Structural models of the OSM-3 kinesin and its mutants

To explore the impact of the elbow phosphorylation on the OSM-3 kinesin, we first predicted a model of the OSM-3 monomer using LocalColabFold and relaxed the mutated models with PyRosetta to calculate the energy-minimized conformations of these mutations (**Fig. 3A**) (Chaudhury et al., 2010; Jumper et al., 2021; Mirdita et al., 2022). The WT and PM models resulted in similar conformations, with their tails binding to the motor head and forming a reverse β -sheet secondary structure. The motor-tail interaction, reported in other motors, typically results in an autoinhibitory state (Coy et al., 1999; Dietrich et al., 2008; Espeut et al., 2008; Friedman and Vale, 1999). In contrast, the G444E mutation, a characterized hyperactive mutation, showed a head-tail dissociation conformation. Similarly, the OSM-3PD protein also exhibited a head-tail dissociation conformational change during relaxation (**Fig. 3A**).

To trace the clues of the conformational change, we evaluated the energy of pre-relaxed structures around the phosphorylated residues (**Fig. 3B-C**, Appendix Table S1). Compared to the PM model of OSM-3, the PD-mutated side chains had lower side-chain rotamer energy (The ‘fa_dun’ term) (Alford et al., 2017), likely due to the larger and higher charged residues in the PM state.

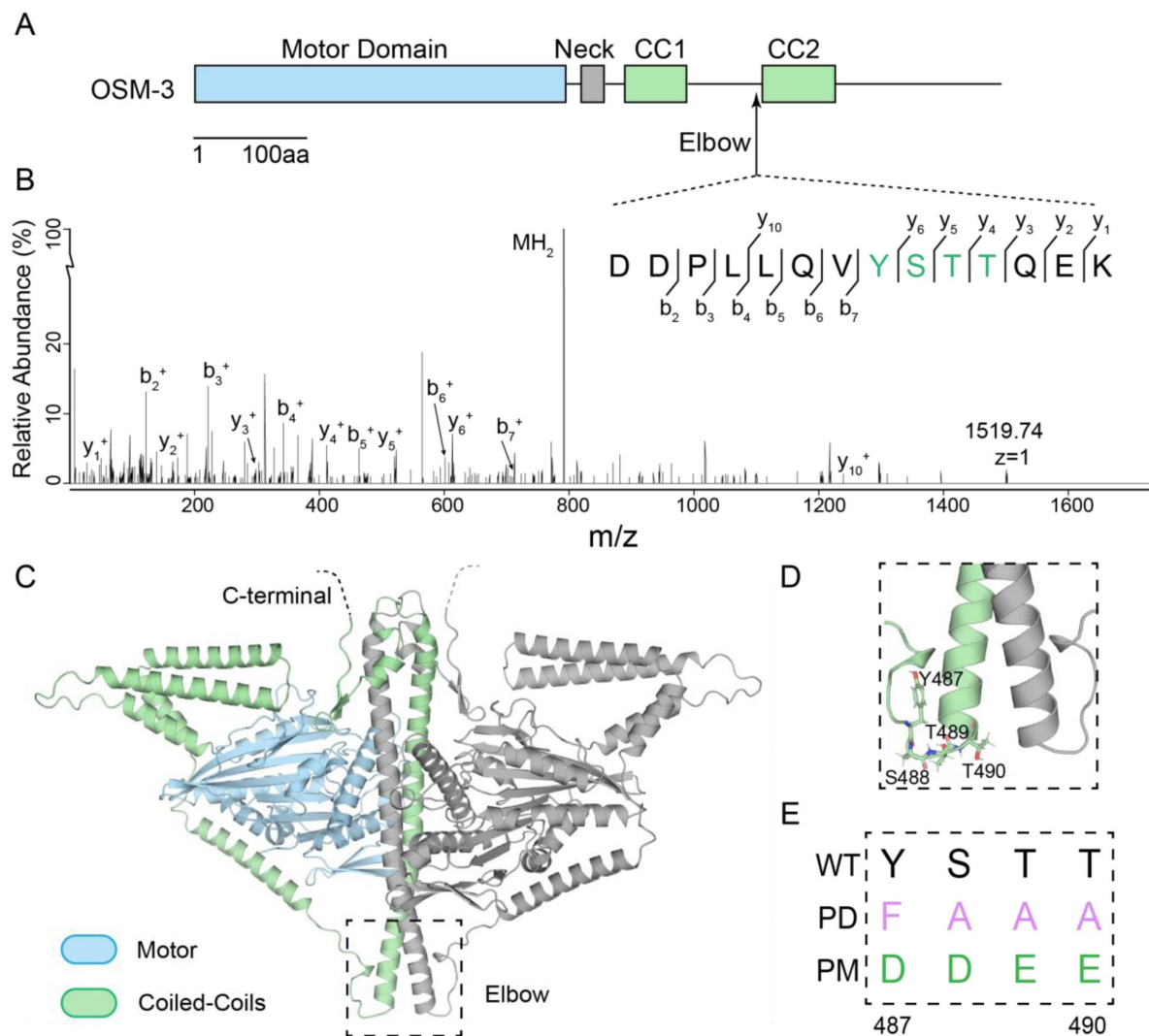


Fig. 1.

NEKL-3 phosphorylates OSM-3 at its "elbow"

(A) Schematic of the full-length OSM-3. Motor domain (blue), neck (gray) and coiled-coils (green) are indicated. CC, coiled-coil. (B) Mass spectrum of an OSM-3 peptide that was phosphorylated by NEKL-3. Phosphorylated gel bands were subjected to MS analysis searching for phosphorylation modifications. Residues 487 to 490 of OSM-3 were phosphorylated and were marked by green color. (C) Phosphorylated 488-490 residues are at the "elbow" of OSM-3. It shows the overall structure of the homodimeric OSM-3 predicted by alphafold2. The dashed square marks the "elbow" region and is zoomed-in in (D). (E) Genome editing constructs of the elbow, showing the PD (phospho-dead) and PM (phosphor-mimic) sequences comparing to wild type. Residue 487-490 of OSM-3 was edited to "FAAA" for PD strain or edited to "DDEE" for PM strain. Abbreviations: Y, Tyr; S, Ser; T, Thr; F, Phe; A, Ala; D, Asp; E, Glu.

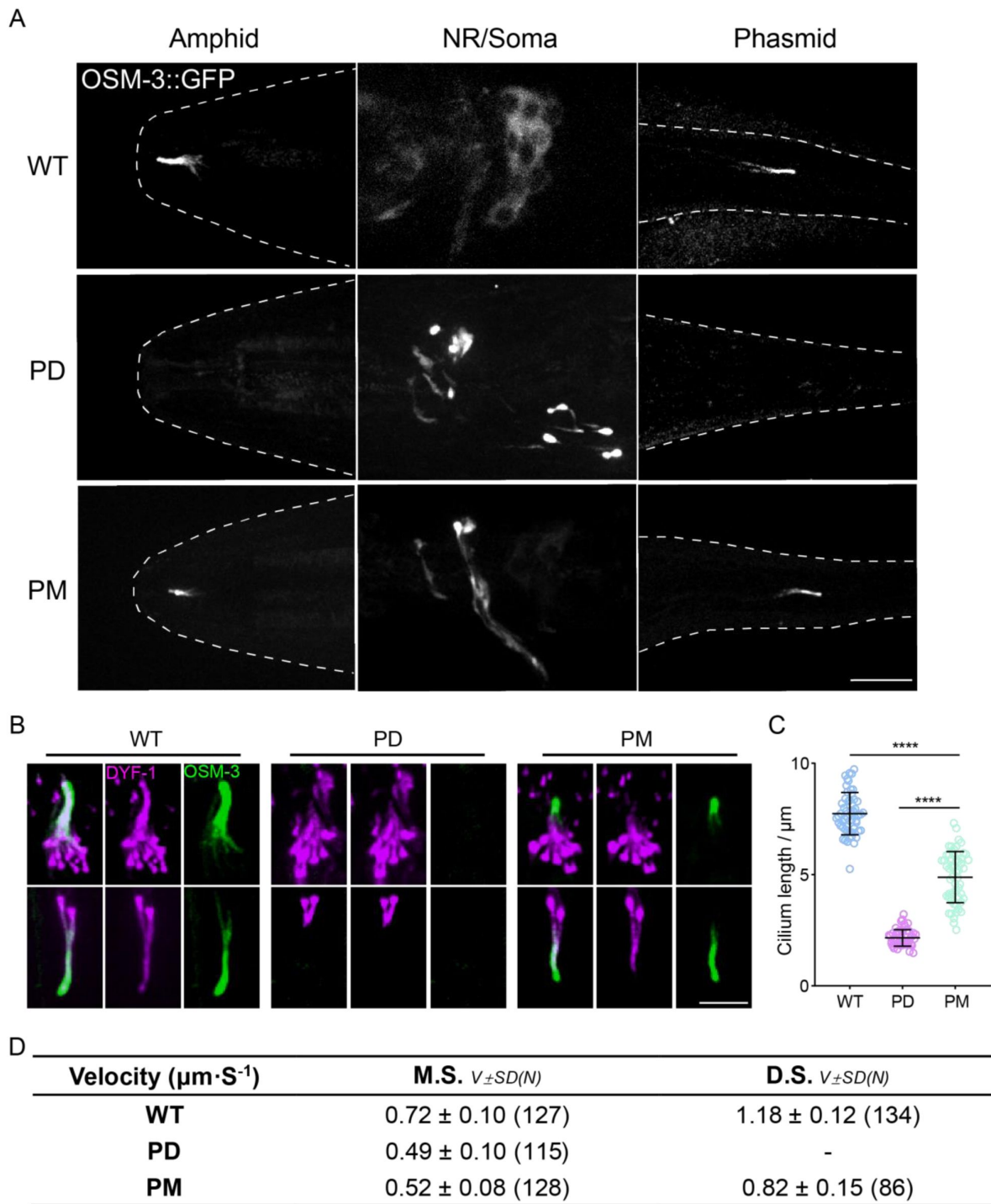


Fig. 2.

Phosphorylation at the elbow serves as an inhibitory post-translational modification

(A) Representative images of the phospho-dead (PD) and phospho-mimic (PM) knock-in worms showing their OSM-3 signal at amphid cilia, amphid neuronal soma and phasmid cilia, respectively. The contours of the worms are marked by white dashed lines. Scale bar, 10 μm . (B) Representative images of the cilia of PD and PM worms marked by the ciliary marker DYF-1::mScarlet. Scale bar, 5 μm . (C) Statistics of the cilium length of the strains showed in (B). The lengths of DYF-1::mScarlet signals were measured and analyzed. (D) IFT velocities of PD and PM worms. ****, $p < 0.0001$, analyzed by one way ANOVA, p values were adjusted by BH method.

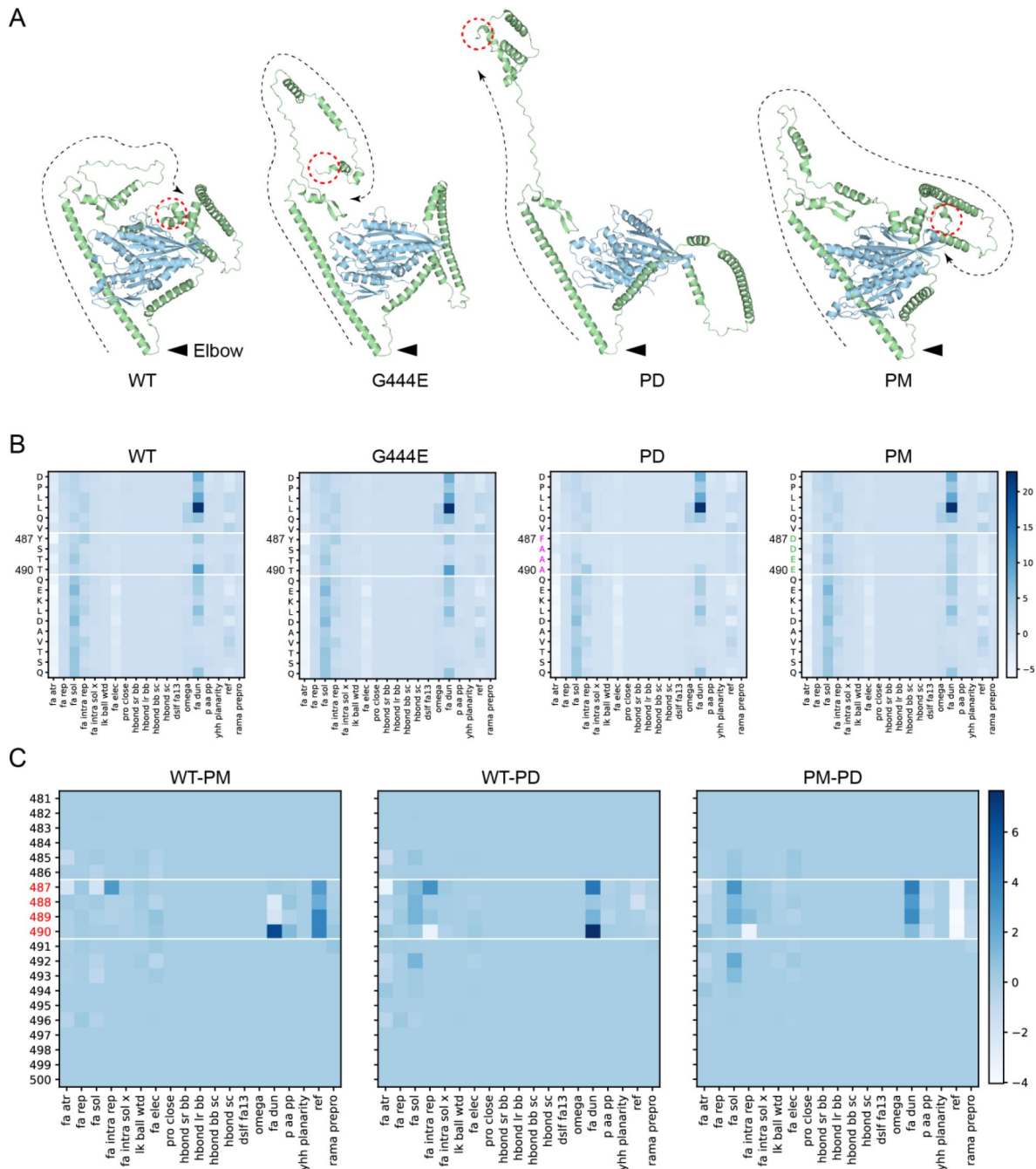


Fig. 3.

Structural models of the OSM-3 kinesin and its mutants

(A) Relaxed structure models of OSM-3 and mutants. Black arrow heads indicate the elbow while red dashed circles mark the C-terminus of the protein. Black dashed lines showed the extending direction from the elbow towards the C-terminus. WT and PM showed close interaction between the tail and motor domain while G444E and PD showed that the tails are faraway from the motor. (B) Heatmaps of the energy states of the pre-relaxed structure models from amino acid 481st to 500th, as labeled on the left; the amino acids between the white lines are the elbow region; each row represents an energy item as labeled on the bottom. (C) Heatmaps comparing the energy states by direct subtraction between the mutants and WT. The PD mutant has lower “fa_dun” energy while has higher “ref” energy than that of the PM mutant.

However, the PD mutation residues exhibited higher folding/unfolding free energy (The ‘ref’ term) (Alford et al., 2017 [↗](#)), indicating an unstable folding state of the mutated loop (**Fig. 3B-C** [↗](#), Appendix Table S1). This instability may explain the observed conformational change leading to the autoinhibition release of OSM-3PD.

Biochemical analyses reveal the role of elbow phosphorylation in OSM-3 motility

Using recombinant proteins purified from bacteria (Fig. S2), we examined the microtubule-stimulated ATPase activity of the WT and mutant OSM-3 variants (**Fig. 4A** [↗](#)). The WT full-length OSM-3 exhibited minimal ATPase activity due to its autoinhibition. As previously reported, the hyperactive OSM-3G444E mutation showed a significant increase in ATPase activity (Imanishi et al., 2006 [↗](#); Xie et al., 2024 [↗](#)). Consistent with our structural predictions, the OSM-3PD mutation also led to an upregulation of ATPase activity to a level similar to OSM-3G444E (**Fig. 4A** [↗](#)). In contrast, OSM-3PM caused a slight increase in ATPase activity compared to WT but was markedly lower than OSM-3G444E or OSM-3PD (**Fig. 4A** [↗](#)).

Next, we performed microtubule gliding assays to examine the gliding activity of OSM-3 and its mutants. In support of the changes in ATPase activities, OSM-3PD exhibited increased gliding speed compared to WT but was slower than OSM-3G444E (**Fig. 4B-D** [↗](#)). To examine the processivity of OSM-3, we used a total internal reflection fluorescence (TIRF) microscope to visualize GFP-labeled motors. We did not detect any movement of WT OSM-3 or OSM-3PM on microtubules (**Fig. 4B, E** [↗](#)-G). In contrast, OSM-3PD, similar to the hyperactive OSM-3G444E, underwent persistent movement along microtubules, albeit at a slower speed and with a lower landing rate (**Fig. 4B, E** [↗](#)-G). These data indicate that the OSM-3PD mutation disrupts the autoinhibition of full-length OSM-3, providing *in vitro* evidence for the inhibitory effect of NEKL-3 phosphorylation at the OSM-3 elbow region in regulating its autoinhibition.

Genetic suppressor screens identified an intragenic suppressor of OSM-3PD

We performed a forward genetic suppressor screen to identify mutations that could restore the ciliary function of *osm-3PD* (**Fig. 5A** [↗](#)). After screening over 10,000 haploid genomes, we isolated two suppressor mutants that exhibited the uptake of the fluorescent dye DiI through cilia, similar to wild-type animals ($n = 100$ for each) (**Fig. 5B-C** [↗](#)). Both suppressors were intragenic and led to the same A489T missense mutation in the elbow region (**Fig. 5B-C** [↗](#)). Whole-genome sequencing data indicated that these two strains carried distinct mutations in many other loci (Appendix Table S2-3), supporting the notion that these two alleles were isolated independently from our suppressor screens.

We studied the *in vivo* behaviors of the OSM-3PD-A489T variant and observed that the vast majority of GFP fluorescence was localized within cilia (**Fig. 5D** [↗](#)). Notably, neither of the GFP-tagged OSM-3PD-A489T mutants displayed any discernible GFP puncta throughout the entirety of the *C. elegans* body ($N > 100$ for each double mutant), indicating that the A489T mutation completely eliminated the aberrant accumulation of OSM-3PD (**Fig. 5D** [↗](#)). By measuring IFT speeds, we showed that OSM-3PD-A489T moved indistinguishably from WT along the middle and distal ciliary segments (**Fig. 5D-E** [↗](#)). These observations were further confirmed in transgenic animals expressing GFP-tagged OSM-3PD-A489T in the *osm-3(p802)* null allele background (**Fig. 5D-E** [↗](#)). Collectively, these *in vivo* findings indicate that the A489T mutation suppresses the defects of OSM-3PD, restoring its normal function and localization.

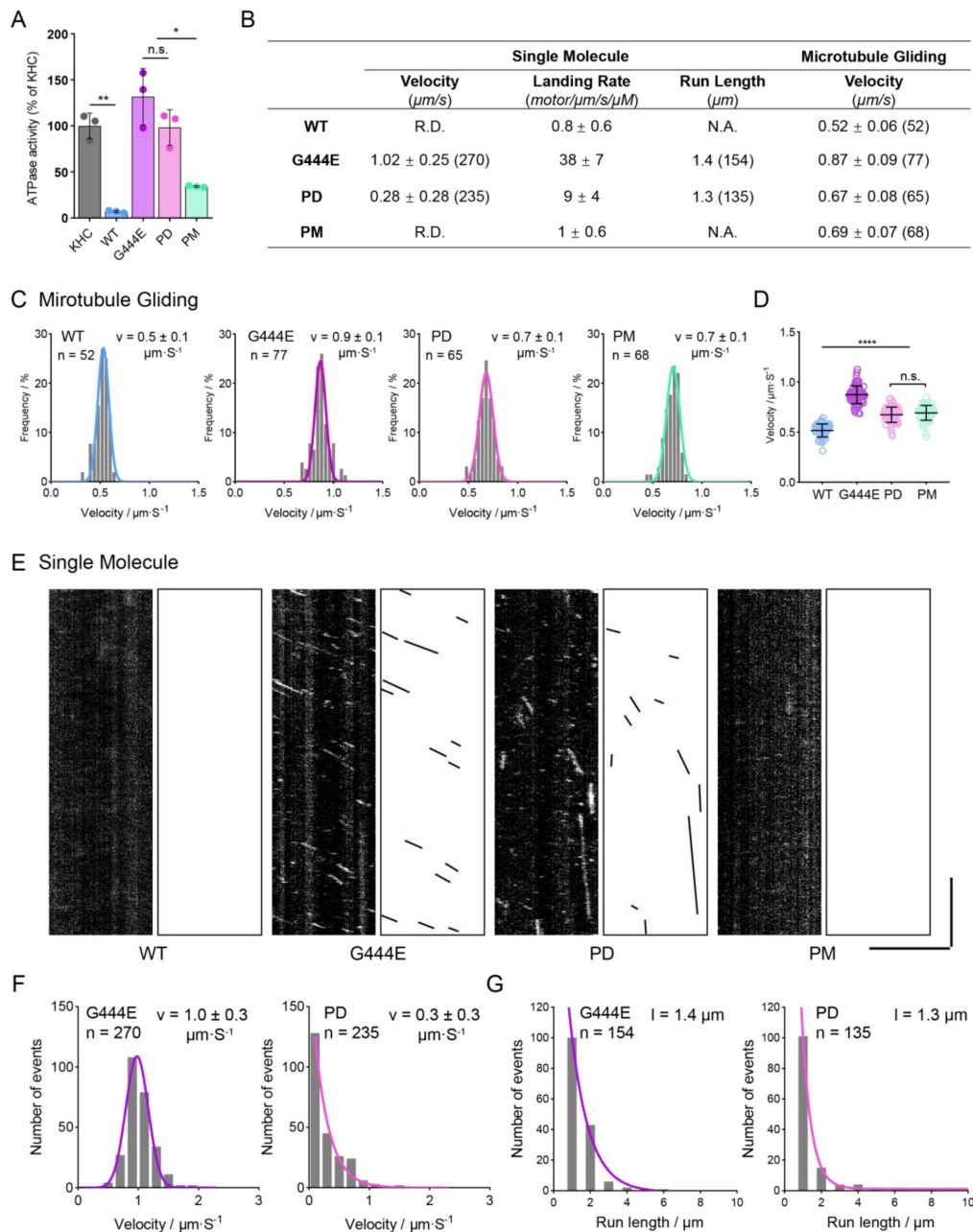


Fig. 4.

Phospho-dead OSM-3 behaves constitutively active in vitro while Phospho-mimic OSM-3 stays autoinhibited.

(A) Representative kymographs of the single-molecular movements of WT OSM-3 and mutants as indicated. G444E, the constitutively active positive control. Scale bars, vertical, 10s; horizontal, 5 μm . (B) Velocity distributions of the single-molecular assays. n , total events measured. v , $\mu\text{m}\cdot\text{s}^{-1}$, average velocity with standard deviation. The distribution of G444E was fitted with a Gaussian distribution curve while the distribution of PD was fitted with a one-phase decay curve. (C) Run length distributions of the single-molecular assays. n , total events measured. l , μm , average run length. The curves were fitted with the Gaussian distribution. (D) Velocity distributions of microtubule gliding assays of the indicated OSM-3 constructs. n , total events measured. v , $\mu\text{m}\cdot\text{s}^{-1}$, average velocity with standard deviation. (E) Statistics of microtubule gliding velocities shown in (D). (F) Microtubule stimulated ATPase activity of WT OSM-3 and mutants. KHC, kinesin heavy chain. Average activity of KHC was set to 100% and others was normalized to KHC. (G) Summary of the single-molecular assay and the microtubule gliding assay. R.D., rarely detected. N.A., not available. Data are [mean $\pm$ SD (number of events)]. *, $p < 0.05$, **, $p < 0.01$, ****, $p < 0.0001$, analyzed by one way ANOVA, p values were adjusted by BH method.

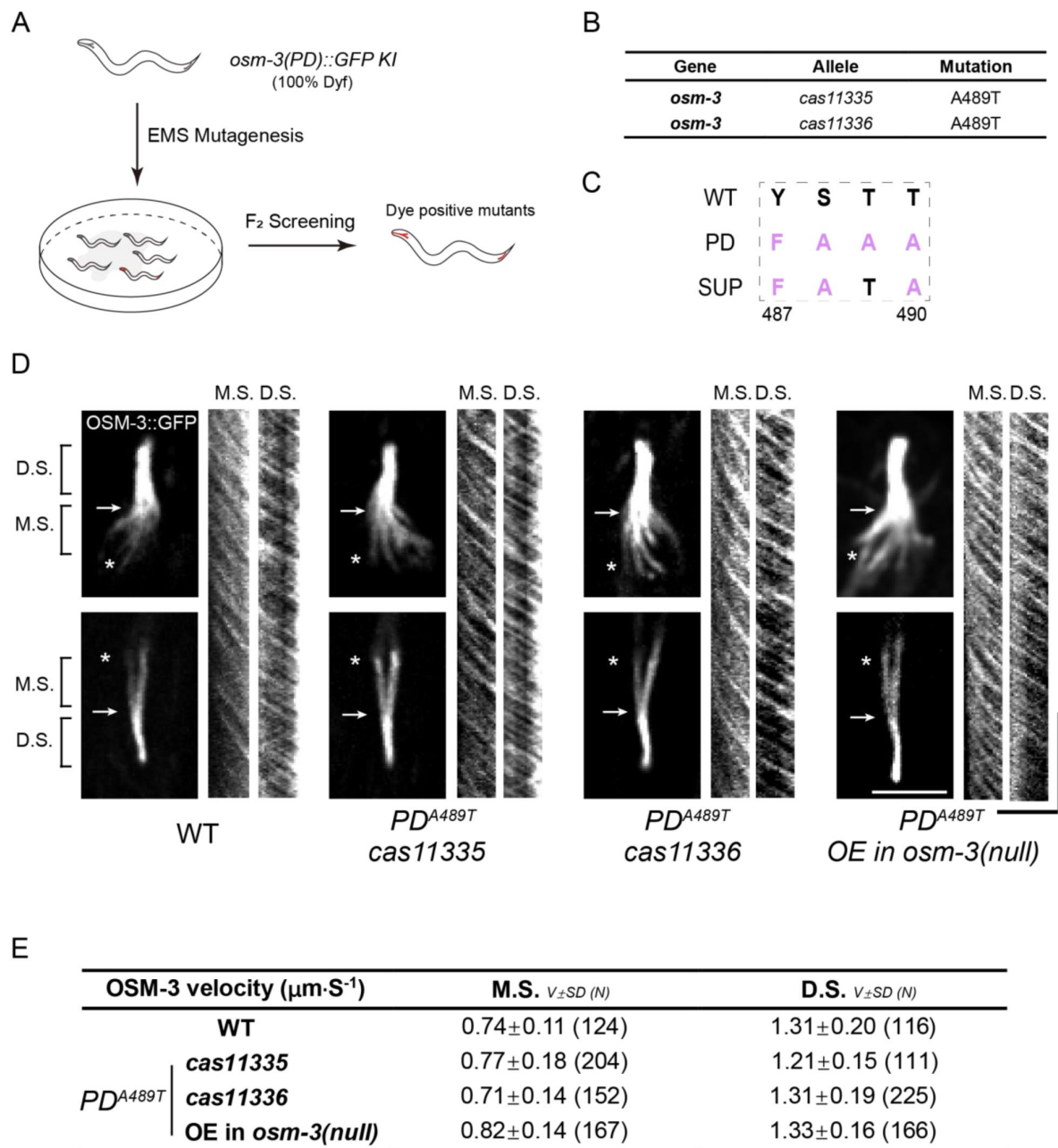


Fig. 5.

Genetic screening identified T489 as the key regulatory residue in the elbow of OSM-3.

(A) Schematics of the forward genetic screen. 100% Dyf *osm-3(PD)::GFP KI* worms were mutated by EMS and F₂ progenies were screened for dye filling positive mutants. (B) Two independent suppressor mutants cloned from the genetic screening. (C) Amino acid sequences of the suppressor mutants that showed the mutations at the elbow. (D) Representative images of the cilia of the suppressors and the kymographs showing the velocity of OSM-3. The rightmost panel shows the same OSM-3 version (487-489: "FATA") with the suppressors but over-expressed under the ciliary Pdyf-1 promoter in *osm-3(p802)* worm. The arrows indicate the junction between middle and distal segments while the asterisks indicate the ciliary base. M.S., middle segment; D.S., distal segment. Scale bars, vertical, 10s; horizontal, 5 μm . (E) Summary of the OSM-3 velocity. M.S., middle segment. D.S. distal segment. Data are [mean $\pm$ SD (number of events)].

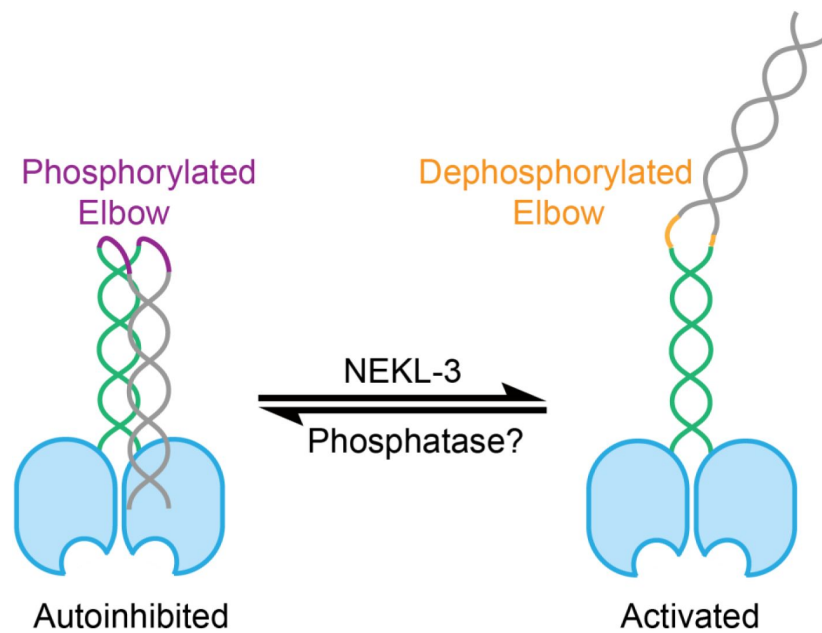


Fig. 6.

Proposed model for elbow regulation of OSM-3 by NEKL-3 phosphorylation

OSM-3 is phosphorylated at the elbow by NEKL-3 and behaved autoinhibited while after dephosphorylation, OSM-3 turns from a compact state to an extended state due to the elbow conformational changes.

Discussion

This study introduces a model that elucidates the essential role of OSM-3 elbow phosphorylation in modulating kinesin motility. We demonstrate that NEKL-3 kinase phosphorylates the elbow region of OSM-3 kinesin *in vitro*. Fluorescence of OSM-3::GFP and NEKL-3::GFP was observed in the soma and dendrites of sensory neurons, indicating the sites of protein synthesis and potential regulatory activity. We propose that OSM-3 is synthesized in the soma, where its elbow region is subsequently phosphorylated by NEKL-3. This phosphorylation is critical to inhibit OSM-3 motility prior to its arrival at the cilia. Previous findings have shown that cytoplasmic dynein-1 facilitates the transport of centrioles from the soma along the dendrites to the dendritic tip, the site of ciliary base formation (Li et al., 2017 [DOI](#)). We hypothesize that inactive OSM-3 is similarly transported, possibly by dynein-1, to the ciliary base as a cargo molecule. Dephosphorylation at the ciliary middle segment appears necessary to activate OSM-3, thereby driving IFT along the ciliary distal segments.

In the absence of phosphorylation at the soma or dendrites, as demonstrated in the OSM-3PD model, OSM-3 may remain constitutively active. This hyperactivity might induce cellular responses that erroneously direct hyperactive OSM-3 to neurite tips rather than to cilia. Furthermore, OSM-3 harboring the G444E mutation within the hinge region exhibits constitutive activity (Imanishi et al., 2006 [DOI](#)). Our recent study revealed that this hyperactive form of OSM-3 was also absent from cilia and was instead expelled through membrane abscission at the tips of aberrant neurites (Xie et al., 2024 [DOI](#)). Adjacent glial cells subsequently engulf and degrade the extruded OSM-3G444E, a process mediated by the engulfment receptor CED-1 (Xie et al., 2024 [DOI](#)). We propose that the hyperactive OSM-3PD is subject to a similar fate, leading to ciliary defects analogous to those observed in the *osm-3* null or *osm-3G444E* alleles.

Our mass spectrometry analysis did not yield conclusive data regarding the specific residue in the elbow region undergoing phosphorylation. However, intriguingly, genetic suppressor screens revealed that the A489T mutation fully restores OSM-3PD localization to the cilia. Although direct mass spectrometric analysis from OSM-3PD-A489T animals is pending, the genetic suppression strongly implicates the role of the T489 site in regulating elbow phosphorylation. Considering the previous proteomic study of *Chlamydomonas* flagellar proteome identified protein phosphatases in cilia (Pazour et al., 2005 [DOI](#)), we postulate that a protein phosphatase may dephosphorylate inhibitory phosphorylations on the OSM-3 elbow. Once within the cilia, this phosphatase in the ciliary middle segment likely dephosphorylates OSM-3, relieving inhibition from both the motor domain and the elbow region, thereby facilitating OSM-3-driven IFT and contributing to the construction of ciliary distal segments.

It is plausible that additional kinases are involved in elbow phosphorylation. Previous studies have shown NEKL-4 can regulate the stability of ciliary microtubules by affecting tubulin glutamylation, indicating the ciliary functions of the NIMA family kinases (Power et al., 2020 [DOI](#); Power et al., 2024 [DOI](#)). NEKL-3 and its homologue NEKL-4 exhibit similar localization patterns in the soma and dendrites. While NEKL-3 is indispensable for OSM-3 regulation, NEKL-4 appears to have a redundant role in this process. Although our phosphorylation assays utilized recombinant NEKL-3 due to technical constraints that we could not generate recombinant NEKL-4 with activity, it remains possible that NEKL-4, akin to NEKL-3, may also phosphorylate the OSM-3 elbow.

This study primarily elucidates the inhibitory phosphorylation of OSM-3 prior to ciliary entry. Equally intriguing is the regulation of OSM-3 at the ciliary tip, where its activity must be curtailed to halt IFT, thus preventing excessive ciliary elongation. Previous studies have identified DYF-5, DYF-18 kinases and other ciliary kinases as crucial regulators limiting ciliary length across various species (Berman et al., 2003 [DOI](#); Burghoorn et al., 2007 [DOI](#); Maurya and Sengupta, 2021 [DOI](#); Omori et

al., 2010 [↗](#); Ozgul et al., 2011 [↗](#); Park et al., 2021 [↗](#); Tucker et al., 2011 [↗](#); Yi et al., 2018 [↗](#)). It is plausible that elbow phosphorylation by these kinases serves as a regulatory mechanism restraining OSM-3 motility at the ciliary tip. Given the ubiquity of the kinesin elbow region across the superfamily, we propose that phosphorylation regulation of this region may represent a widespread mechanism governing kinesin motility. This suggests a fundamental role for elbow phosphorylation in modulating the activity of kinesins, potentially impacting a broad array of cellular processes.

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Additional information

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Author contributions

G. Ou, G. Chen, Z. Zhu, S. Xie and T. Zhou conceived the project. G. Chen performed the experiments, collected and analyzed the data under the supervision of G. Ou. Z. Zhu prepared recombinant NEKL-3 and performed OSM-3 phosphorylation experiments. Z. Guo performed the energy minimization experiments. G. Ou and G. Chen wrote the manuscript and others revised the manuscript.

Competing interests

Authors declare that they have no competing interests.

Data and materials availability

All data are available in the main text or the supplementary materials.

Supplementary Materials

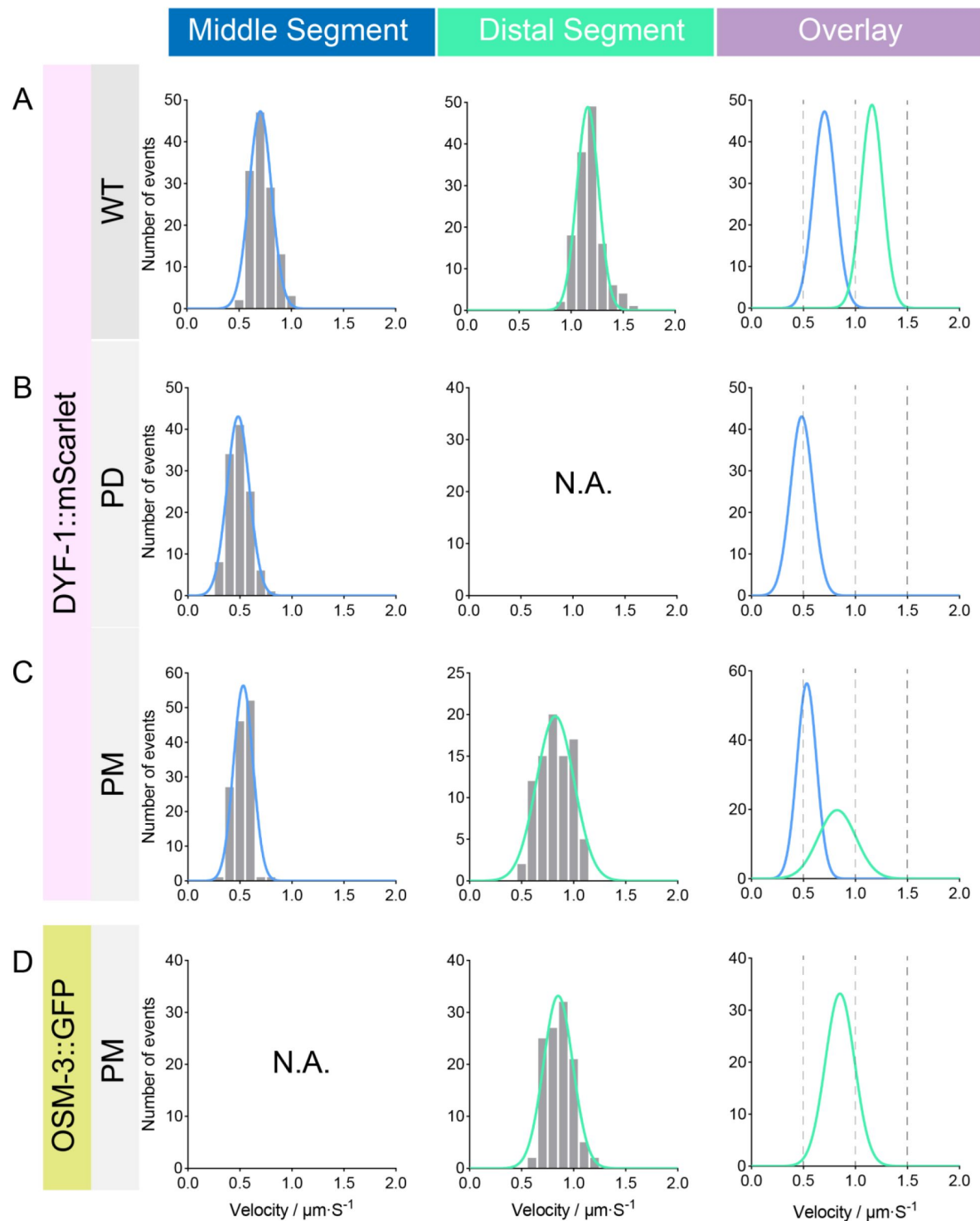


Figure S1.

Statistical analysis of the IFT velocities in *osm-3pd* and *osm-3pm* worms, corresponds to Fig. 2D

(A-C) IFT velocity marked by DYF-1-mScarlet. Genotypes are shown on the left. Left panel, frequency distribution of IFT particles in the middle segment. Middle panel, frequency distribution of IFT particles in the distal segment. Data were fitted with a Gaussian distribution. Right panel, overlay of the fitted curves of middle and distal segments. (D) OSM-3 velocity in *osm-3pm* worms. N.A., not available.

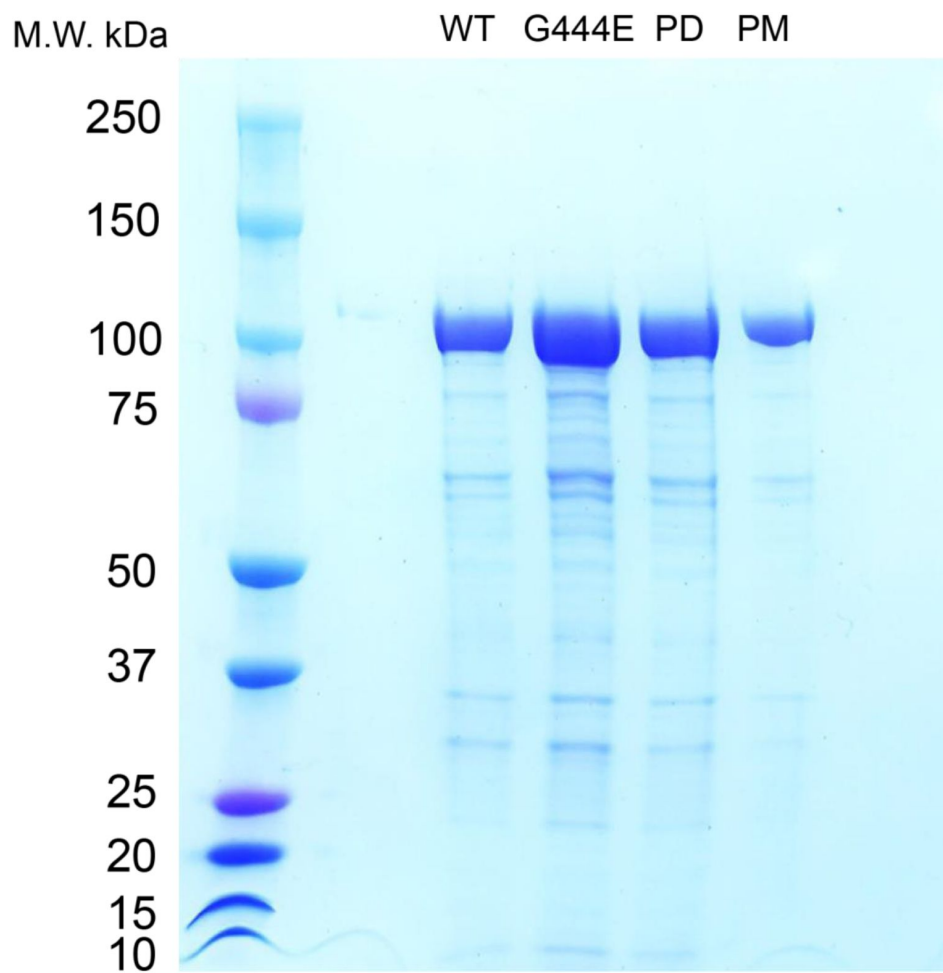


Figure S2.

SDS-PAGE of purified recombinant OSM-3 mutants

SDS-PAGE of the purified recombinant OSM-3::eGFP and mutants, Coomassie Blue stained. The elution peaks of each recombinant protein were shown, labeled on the top.

Methods

C. elegans strain culture

All *C. elegans* strains used in this study were cultured on OP50 seeded NGM (nematode growth medium) plates at 20 °C. Appendix Table S4 summarizes the strains.

Molecular biology

The knock-in *osm-3-pm* and *osm-3-pd* strains were generated by SunyBiotech in the *osm-3::GFP KI* (*SYD0199*) background by CRSIPR-Cas9 based methods and confirmed by Sanger sequencing.

For OSM-3 transgenic strains, point mutation constructs were generated by PCR and the desired constructs were co-injected with *rol-6[su1006]* into the germ line of young adult hermaphrodites. Marker-positive F1s were singled and the F2s with transgenes were identified as transgenic lines. Two independent lines were maintained and used for experiments.

Live imaging

Live imaging was performed as described (Xie et al., 2024 [DOI](#)). Basically, young adult worms were anesthetized with 0.1 mmol/L levamisole in M9 buffer and fixed on a 3% agarose pad. For spinning disk confocal system, imaging was performed on an Axio Observer Z1 microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 100×, 1.49 NA objective, an electron-multiplying (EM) charge-coupled device (CCD) camera (Andor iXon+ DU-897D-C00#BV-500), and the 488/561 nm lines of a Sapphire CW CDRH USB Laser System attached to a spinning disk confocal scan head (Yokogawa CSU-X1 Spinning Disk Unit). Images were acquired by μ Manager ([https:// www.micro-manager.org](https://www.micro-manager.org) [DOI](#)) at an exposure time of 200ms and analyzed with ImageJ software.

Structure prediction and energy minimization

OSM-3 model was predicted using LocalColabFold (Jumper et al., 2021 [DOI](#)). Mutated proteins were designed by Pymol 2.6, choosing the rotamer of the mutated residues in G444E, PM and PD models with the least clash as the initial conformation.

Then the initial models were subjected to Pyrosetta (Chaudhury et al., 2010 [DOI](#)). The energies of pre-relaxed models were evaluated with Rosetta Energy Function 2015 (Alford et al., 2017 [DOI](#)), and then the relax procedure were applied to the models with default parameters to obtain the relaxed models visualized by Pymol to minimize the energy of these models.

Protein preparation

Protein preparation was performed as described (Xie et al., 2024 [DOI](#)). Basically, point mutations was introduced in to pET.M.3C OSM-3-eGFP-His₆ plasmid for prokaryotic expression. Plasmid transformed *E.coli*(*BL21*) was cultured at 37°C and induced overnight at 23°C with 0.2 mM IPTG. Cells were lysed in lysis buffer (50 mM NaPO₄ pH8.0, 250 mM NaCl, 20 mM imidazole, 10 mM bME, 0.5 mM ATP, 1 mM MgCl₂, Complete Protease Inhibitor Cocktail (Roche)) and Ni-NTA beads were applied for affinity purification. After incubation, beads were washed with wash buffer (50 mM NaPO₄ pH6.0, 250 mM NaCl, 10 mM bME, 0.1 mM ATP, 1 mM MgCl₂) and eluted with elute buffer (50 mM NaPO₄ pH7.2, 250 mM NaCl, 500 mM imidazole, 10 mM bME, 0.1 mM ATP, 1 mM MgCl₂). Protein concentration was determined by standard Bradford assay.

ATPase assays

Microtubule stimulated ATPase activity assays were performed with a commercial kit (HTS Kinesin ATPase Endpoint Assay Biochem Kit, Cytoskeleton Inc.) following the manufacturer's instructions.

In vitro Motility assays

And the assays were performed as described. Briefly, the elution peak fraction was applied to a Zeba™ Spin Desalting Column to exchange the protein into motility buffer (80mM pipes-K pH6.8, 200mM KCl, 1mM EGTA, 2 mM MgCl₂, 0.1 mM ATP, 10 mM bME) before use. For microtubule gliding assays, OSM-3 was flowed into a flow cell in desired concentration and rhodamine labeled microtubules were subsequently flowed-in with assay buffer (BRB80, 1 mM ATP/Mg²⁺, 1% β-Mercaptoethanol, 0.08mg/mL glucose oxidase, 0.032 mg/mL catalase and 80 mM glucose). For single molecular assays, microtubules were attached to the flow cell surface via antibodies (SAP4G5), and the motors were flowed-in in a desired concentration in assay buffer. The eGFP and the rhodamine were illuminated by 488 or 561 nm laser at 20 mW, and signals were visualized by Olympus IX83 microscopy equipped with a 150× (1.45 NA, oil, Olympus) objective lens and an ORCA-Flash4.0 V3 camera. The system was controlled by Micro-Manager 2.0.

Genetic screening

To isolate suppressors of *osm-3(PD)*, L4 worms were collected in 4 mL M9 and treated with 20 μL ethyl methanesulfonate (EMS) for 4 hours. Adult F2 animals were subjected to Dye-Filling Assay and Dye-positive mutant animals were individually cultured. Dye (DiI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; Sigma-Aldrich, St. Louis, MO, USA) was used at a final concentration of 20 μg/mL. Whole-genome sequencing was applied to all suppressor strains to identify candidate genes.

Quantifications and statistical analysis

ImageJ software was used to perform measurements and quantifications of the images. For cilium length, the indicated numbers of phasmid cilia were measured. For IFT velocity, the indicated numbers of IFT particles in amphid and phasmid cilia were randomly selected for the measurement. Microtubule-stimulated ATPase activities were derived from three assays and the average activity of kinesin heavy chain (KHC) was set to 100%. Single molecule and gliding data were measured using ImageJ software and all the events measured were selected randomly. Statistical analysis were performed in GraphPad Prism. The statistical differences were determined by two-tailed Student's t-test or ANOVA analysis as described in the figure legends. The frequency distribution of IFT velocity and GFP lifetime was analyzed and fitted with a Gaussian distribution curve.

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Reviewer #1 (Public review):

Summary:

This manuscript is a focused investigation of the phosphor-regulation of a *C. elegans* kinesin-2 motor protein, OSM-3. In *C. elegans* sensory ciliary, kinesin-2 motor proteins Kinesin-II

complex and OSM-3 homodimer transport IFT trains anterogradely to the ciliary tip. Kinesin-II carries OSM-3 as an inactive passenger from the ciliary base to the middle segment, where kinesin-II dissociates from IFT trains and OSM-3 gets activated and transports IFT trains to the distal segment. Therefore, activation/inactivation of OSM-3 plays an essential role in its ciliary function.

Strengths:

In this study, using mass spectrometry, the authors have shown that the NEKL-3 kinase phosphorylates a serine/threonine patch at the hinge region between coiled coils 1 and 2 of an OSM-3 dimer, referred to as the elbow region in ubiquitous kinesin-1. Phosphomimic mutants of these sites inhibit OSM-3 motility both in vitro and in vivo, suggesting that this phosphorylation is critical for the autoinhibition of the motor. Conversely, phospho-dead mutants of these sites hyperactivate OSM-3 motility in vitro and affect the localization of OSM3 in *C. elegans*. The authors also showed that Alanine to Tyrosine mutation of one of the phosphorylation rescues OS-3 function in live worms.

Weaknesses:

Collectively, this study presents evidence for the physiological role of OSM-3 elbow phosphorylation in its autoregulation, which affects ciliary localization and function of this motor. Overall, the work is well performed, and the results mostly support the conclusions of this manuscript. However, the work will benefit from additional experiments to further support conclusions and rule out alternative explanations, filling some logical gaps with new experimental evidence and in-text clarifications, and improving writing.

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Reviewer #2 (Public review):

Summary:

The regulation of kinesin is fundamental to cellular morphogenesis. Previously, it has been shown that OSM-3, a kinesin required for intraflagellar transport (IFT), is regulated by autoinhibition. However, it remains totally elusive how the autoinhibition of OSM-3 is released. In this study, the authors have shown that NEKL-3 phosphorylates OSM-3 and releases its autoinhibition.

The authors found NEKL-3 directly phosphorylates OSM-3 (although the method is not described clearly) (Figure 1). The phosphorylated residue is the "elbow" of OSM-3. The authors introduced phospho-dead (PD) and phospho-mimic (PM) mutations by genome editing and found that the OSM-3(PD) protein does not form cilia, and instead, accumulates to the axonal tips. The phenotype is similar to another constitutive active mutant of OSM-3, OSM-3(G444A) (Imanishi et al., 2006; Xie et al., 2024). *osm-3*(PM) has shorter cilia, which resembles with loss of function mutants of *osm-3* (Figure 3). The authors did structural prediction and showed that G444E and PD mutations change the conformation of OSM-3 protein (Figure 3). In the single-molecule assays G444E and PD mutations exhibited increased landing rate (Figure 4). By unbiased genetic screening, the authors identified a suppressor mutant of *osm-3*(PD), in which A489T occurs. The result confirms the importance of this residue. Based on these results, the authors suggest that NEKL-3 induces phosphorylation of the elbow domain and inactivates OSM-3 motor when the motor is synthesized in the cell body. This regulation is essential for proper cilia formation.

Strengths:

The finding is interesting and gives new insight into how the IFT motor is regulated.

Weaknesses:

The methods section has not presented sufficient information to reproduce this study.

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