SUPPLEMENTARY INFORMATION APPENDIX (SI APPENDIX)

ON and OFF Controls within Dynein-Dynactin on Native Cargoes
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Antibodies

Mammalian-DIC (Santacruz Sc13524), P150 (BD Biosciences 612709), Actin (Cytoskeleton AAN01), Tubulin (Invitrogen 236-10501), Rab7 (Cell Signaling 9367), NudE (Santacruz Sc100328) *Dictyostelium*-Dynein (generated against Dynein stalk head)(29), Dynactin (generated against CC1 domain) , Rab7 (peptide antibody)(29), CAPGly (details below), actin (Abclonal AC009) His (Sigma H1029), Rabbit IgG (Biobharti BB-AB002), GST (Cloud Clone TAX158Ge22), FLAG (Sigma F3165). For western all antibodies were used at 1:3000 dilution. For imaging experiments, anti-His was used as 1: 400 dilution.

Cloning and protein purification

DIC proteins- For DIC WT, 1-126 amino acids of *Dictyostelium* DIC was cloned between BamHI and XhoI sites into pET22b expression vector containing a C-terminal His tag. For DIC Mutant, 79th serine residue was mutated to aspartate (S79D) using Q5 site directed mutagenesis kit (New England Biolabs). Both *Dictyostelium* DIC WT and Mut proteins as well as mammalian DIC-WT (S84A) and DIC-Mut (S84D) (kind gift from K. Vaughans lab) were purified from LIMO cells (Invitrogen) by inducing 1 liter culture with 0.5mM IPTG overnight at 22 °C. Cell pellet was lysed using lysis buffer (20mM Tris, 150mM NaCl, 1mM DTT pH 7.4 with 1% Triton) complemented with protease inhibitors - 1X PIC (Roche) and 1mM PMSF (Sigma T8830). The lysate was incubated with 1mg/ml lysozyme (Sigma 62971) at 4°C for 30 mins and further sonicated 5 times for 10 sec with 30 sec intervals on ice. After lysis, the debris and membrane were pelleted. The clarified lysate was then incubated with 1 ml Ni-NTA beads (Qiagen) for 3 hours at 4°C, flow through was collected and beads were washed with 10 column volumes of wash buffer (20mM Tris, 300mM NaCl, 1mM DTT pH 7.4). Finally, elution of bound proteins was done with 400mM imidazole containing lysis buffer (pH 8). Eluted proteins were dialyzed in PBS and aliquoted and stored at -80°C for use.

CAPGly protein and Antibody: First 78 residues of the CAPGly domain of *Dictyostelium* P150 were cloned between NdeI and HindIII sites of pET22b expression vector. Protein was purified by transforming in BL21 (DE3) cells and induced with 0.4mM IPTG for 4 hours at 20°C. CAPGly protein was purified in a manner similar to DIC proteins, dialyzed and stored at -80°C.

Purified CAPGly protein was used to generate a polyclonal antibody raised in rabbit (Bioklone, India) against *Dictyostelium* P150.

CC1 protein- *Dictyostelium* CC1 of P150 was cloned using genomic DNA into BamHI and SalI sites of pGEX4T3 vector. Protein was purified from BL21 (DE3) cells and induced with 0.5mM IPTG for 4 hours at 25°C. GST-CC1 was purified in a manner similar to other proteins except that we used Glutathione beads (GE Healthcare) for binding GST-CC1 and the protein was eluted with reduced glutathione (40mM).

Mammalian CC1:- CC1 in pVEX vector was obtained from Trina Schroer. pVEX CC1 was transformed in BL21(DE3) cells and induced with 0.5mM IPTG overnight at 25°C. Cells were lysed by sonication followed by centrifugation at 37,000rpm for 45 min. Protein precipitation was done using 25% w/v ammonium sulphate. The protein precipitated was collected by centrifugation at 5000g for 30mins at 4°C. The protein precipitate was resuspended in PBS having EDTA and DTT and 0.75M KCl followed by boiling at 100°C for 3 mins. The boiled sample was centrifuged at 5000g for 30 min at 4°C. The supernatant was decanted and passed through 0.2 µM filter and loaded

onto a column. Mono Q column (Amersham) was connected to the AKTA machine and washed with 20% ethanol. Next, the column was washed with water, followed by 0.2N NaOH followed by 0.2N HCl. Finally the column was washed with around 200ml water, till the UV plot was constant. Column was equilibrated with 10 volumes of PBS pH 7.2**.** Protein was injected in the column once the column was equilibrated. The flow rate was set to 2ml/min. After that, washing was done with PBS to remove any non-specific proteins. A gradient of 0-1000mM KCl in 20mM Tris-HCl was used to elute the protein**.** Samples with maximum UV absorbance were collected and concentrated using Amicon centricon tube, dialysed in PBS and stored in -80°C.

Dynein MT1 domain –*Dictyostelium* dynein microtubule binding domain (MT1) construct in pET14b vector was obtained from Michael Koonce (1). This is essentially the domain of dynein that binds to MTs. Protein purification was carried out as mentioned for DIC proteins.

MT pelleting assay was adapted from (2) and (3). Either rat brain or *Dictyostelium* high speed supernatant (HSS) was used. Goat brain (5 gram of brain after fat removal) or *Dictyostelium* cell pellet (from 200 ml cultured cells) were lysed mechanically using Dounce homogenizer (goat brain) or by passing through 5µm syringe filter (*Dictyostelium*). The crude lysate was spun at 200000*g* for 30 mins over a 25% sucrose gradient to obtain HSS or cytosol. For each condition, cytosol was treated with either mammalian or *Dictyostelium* DIC WT or mutant proteins (0.25µM) and with IgG or *Dictyostelium* anti-CAPGly antibody (0.5µM) on ice for 30 min. Following treatment, motors from cytosol were allowed to bind with *in vitro* polymerized 0.5mg/ml taxol stabilized MTs. To measure dynein ON rate, the unbinding/detachment of motors was prevented by depleting the ATP in cytosol by adding 3mM glucose, 15U/ml hexokinase, 4mM $MgCl_2$ to cytosol. To measure dynein OFF rate, the experiment was performed in presence of cytosolic ATP. Finally, cytosol+MT mix is loaded onto a 25% sucrose gradient with 20 µM taxol and MTs along with bound motors are pelleted at 90,000g for 20 min. The glassy microtubule pellet is gently washed twice with PBS without dislodging it and finally resuspended in PBS containing 0.1% SDS using a cut tip. After protein estimation, the resuspended MT solution was analysed by western blotting to assay for the amounts of dynein motors bound to MTs. To detect binding of recombinant Dynein-MT1 to MTs, 1mg/ml of His tagged protein was incubated with IgG or anti-CAPGly antibody for 30 min on ice in presence of *in vitro* polymerized MTs. MTs were pelleted and the pellet was examined for dynein MTBD binding using western blotting.

Determining DD levels on membrane fraction- For assaying DD amounts on membrane fraction, *Dictyostelium* (100ml culture) or RAW cells at 80% confluency (5X100mm plates) were pelleted at low speed. Pellet was resuspended in buffer containing 20mM Tris and 4mM EGTA with 5% sucrose. Cell pellet was mechanically lysed either by passing through a 5 µm syringe filter (in case of *Dictyostelium*) or using Dounce homogenizer (for RAW cells). The crude lysate was spun at 1100*g* for 10 mins to pellet the nucleus and un-lysed cells. Supernatant was collected as membrane fraction along with cytosol. This fraction was treated with 0.5 µM DIC-WT or DIC Mutant proteins on ice for 15 min, overlaid on a cushion containing 25% sucrose and subjected to a high speed spin at 200000*g* for 20 min. Thus, soluble proteins from cytosol including unbound DIC proteins remain in the top supernatant fraction while the membranes with bound proteins are pelleted at the bottom. The pellet fraction was resuspended in PBS buffer containing 0.1% triton and dynein and dynactin levels were assessed by western blotting.

Treatment and Immuno-staining of Phagosomes- Latex or silica bead phagosomes were extracted from cells (RAW or *Dictyostelium* cells). Phagosomes were treated with 0.25µM DIC-WT or DIC Mutant proteins for 10 min on ice, followed by pelleting phagosomes at 20,000g for 5 min. Pellet was washed twice with 200µl of PBS and finally resuspended in 20µl solution. This phagosome solution was spread on polylysine coated coverslips following which staining was performed using the protocol described in Ref. 21 of main manuscript. To detect recombinant

DIC-WT or Mut proteins, phagosomes were stained with anti-His antibody (used at 1:400 dilution) and a secondary mouse antibody (1:400 dilution).

Phagosome preparation, Motility assays and Optical trapping.
Dictyostelium cells were cultured in HL5 medium (ForMedium) with 100µg/ml penicillin-streptomycin (Gibco). Latex bead phagosomes for motility assays were prepared as described (4). In short, *Dictyostelium* cells were incubated with 750nm polystyrene beads (Polysciences 07759) and chased (5 min chase for EPs; 45 min for LPs) at 22°C. The cells were lysed, the lysate was centrifuged, and phagosomes were collected along with the high-speed supernatant and frozen. Each aliquot of phagosome was either untreated (control) or treated with 0.25µM DIC (WT/ Mut) or with 0.5µM IgG or anti-CAPGly antibody. Treatment was done for 10 min on ice before flowing in samples on MTs stuck coverslips for assaying motion.

Polarity labelled microtubules were prepared as described (Ref. 30 of main manuscript). Microtubules were adhered to a coverslip using poly-L-lysine. All motility experiments were done at 1mM ATP concentration in presence of an ATP regenerating system. Motion was observed using differential interference contrast microscopy (DIC) with a 100X, 1.4 NA oil objective (Nikon). Image acquisition was at video rates (30 frames/sec). The optical trapping setup and force measurement techniques have been described (5). The trap stiffness was adjusted between 0.05pN/nanometer to 0.15 pN/nanometer, depending on the experiment. A quadrant photodiode (QPD) was used to obtain stall force records. QPD data was digitized at 2 kHz. Thermal fluctuations were recorded at 40 kHz. For measuring force the video-matching method was used (5).

Estimating fold-excess of recombinant proteins (DIC-WT, DIC-Mut) compared to endogenous DIC on phagosomes- Similar to motility assay, LPs collected in cytosol were treated with 0.25µM DIC-WT protein. 10µl of this mixture (Cytosol-LPs) sample was collected for western. After 10 min incubation on ice, LPs from cytosol were pelleted at 20,000g for 10 min and 10µl of supernatant sample (Cytosol-LPs) was collected. The difference in amount of DIC-WT between the two samples corresponds to the amount of DIC-WT that is bound to phagosomes. In order to determine how much DIC-WT bound to LPs, a standard curve was plotted for different dilutions of purified DIC-WT protein, such that values for both samples (Cytosol+LPs) and (Cytosol-LPs) fall within the curve (Fig S4C). On estimating the amount of DIC-WT protein in both samples, as shown in Fig S4C, we find (Cytosol+LPs) = 0.38 µg and (Cytosol-LPs) = 0.32 µg. It was therefore found that ~84% of protein (=[0.32/0.38]×100) was present in cytosol and 16% remained bound to phagosomes, which corresponds to (0.38-0.32)µg = 60ng of protein in 10µl sample. Our motility mixture has a total volume of 100µl containing cytosol and LPs. Therefore, approximately 600ng protein, i.e $\sim 10^{13}$ molecules of DIC-WT are bound to LPs. Using optical density, we determined the total number of LPs in 100µl reaction = 4×10^{8}. In our previous study (Ref. 29 in main manuscript), we have shown that each LP harbors ~240 dyneins. Because each dynein dimer contains two DIC units, there are ~480 endogenous DIC molecules per LP. This yields a total of $480\times4\times10^{8} \approx 2\times10^{11}$ endogenous DIC molecules on LPs used in a typical motility assay. We therefore have 10^{13} DIC-WT proteins competing against 2×10^{11} endogenous DIC molecules our motility assay. This is an excess of ~50 fold of the recombinant protein, and ensures that most (if not all) DD complexes are affected by DIC-WT on phagosomes.

Treatment of mammalian DDB complexes with DIC proteins
DDB complex from goat brain cytosol was formed using recombinant FLAG tagged BicD. These complexes were pulled down using FLAG antibody beads. The FLAG bead-containing pellet was treated with either mDIC-WT or mDIC-Mut for 20 min on ice. Unbound proteins were removed by washing the pellet three times with PBS. The DDB complex with bound proteins was eluted with SDS dye. Levels of dynein, dynactin and recombinant DIC proteins that co-pelleted with BicD were assayed by western blotting.

Liposome preparation: Liposomes were prepared by freeze-thaw method as described (6,7). In brief, lipids pertaining to 1mM of total lipid concentration were dried in a glass vial using a stream of nitrogen, and then in a vacuum desiccator overnight at room temperature. For 1ml of 5% PA liposome preparation, 3.54µl of 10 mg/ml PA (P9511, Sigma) and 29.26µl of 35mg/ml (850375P, Sigma) were used. Post desiccation, the lipid film was hydrated using 1ml of autoclaved double-distilled water by vortexing vigorously for about 20 minutes with intermittent pauses. This led to the formation of a turbid solution. This mixture of liposomes was transferred to a 1.7ml siliconized polypropylene microcentrifuge tube (T3406, Sigma) and given five cycles of freeze-thaw. Liquid nitrogen was used to freeze the liposomes and the thawing was done at 55°C in a water bath. The liposomes were allowed to cool down to room temperature, then stored at 4°C and used within a few days.

SLB preparation: 500nm carboxylated latex beads (Polysciences 09836) were used for SLB preparation. To avoid clumping, the beads were diluted using water and sonicated in a bath sonicator for at least 20 minutes. The beads were brought back to the original concentration by pelleting at 10,000g for 5 minutes and then resuspended in double-distilled water. For the preparation of SLBs for motility experiments, 5µl of latex beads, 1µl of 1M NaCl, 69µl of autoclaved double-distilled water and 20µl of the above-prepared liposomes were taken together in a 1.7ml siliconized polypropylene microcentrifuge tube (T3406, Sigma) and vortexed for 30 minutes with intermittent rest (1-minute vortexing with 5 minutes rest). The mixture was washed thrice by the addition of 1ml of double-distilled water. SLBs were pelleted at 10,000g for 5 minutes and then finally resuspended in 200µl of double-distilled water. Using the OD at 600nm, the SLB concentration was found to be about $5*10^5$ per µl. The SLBs were stored at room temperature during the experiment and used within 10 hours of preparation. Fresh SLBs are prepared on the day of each experiment. Validation of the SLB preparation was done by assaying for red fluorescence due to incorporation of 0.5 mole % Rhodamine-PE while drying lipids (Fig S6D).

ATP releasate preparation for *Dicytostelium* motors-This protocol is adapted from (2). Motors were purified from *Dictyostelium* cytosol using MT pelleting assay. *Dictyostelium* cytosol was incubated with taxol stabilized MTs (0.5mg/ml) for motors binding. ATP from cytosol was depleted by addition of 3mM glucose, 15U/ml hexokinase, 4mM AMP-PNP and 4mM $MgCl_2$. Bound motors were co-pelleted with MTs over a 25% sucrose cushion at 90,000g for 20 min. Active motors were released from MT by addition of 10mM (ATP + $MgCl_2$) following which motor fraction is present in supernatant while MTs with un-released motors are pelleted. The supernatant is called ATP releasate fraction and was stored in aliquots in liquid nitrogen.

SLB treatment for motility – SLBs were incubated with motor releasate fraction (typically 3µl SLBs incubated with 0.5-1µg of releasate fraction) for 10 min on ice followed by incubation of 0.5µM IgG or anti-CAPGly antibody. The mixture was diluted to 30µl using 30mM Tris pH 8 buffer containing 25% sucrose and was finally supplemented with ATP regenerating system before assaying motility.

Dot blot assay - To detect binding of DIC-WT and Mut proteins with Dynactin P150 (CC1 domain), control protein GST or GST-tagged CC1 were spotted on a nitrocellulose membrane. Once the spotted proteins were completely dried, the membrane was blocked and overlayed with DIC WT or DIC Mut proteins (1mg/ml) overnight. The membrane was washed with PBS with 0.05% tween, blocked with 5% milk in PBS and probed with anti His antibody to detect His tagged DIC WT or Mut proteins bound to CC1. To ensure equal loading of GST and CC1-GST proteins, the membrane without any overlay was directly probed with GST antibody. To detect DIC-NudE interaction, His tagged mDIC-WT and mDIC-Mutant (S84D) were spotted on a membrane. In this case, the membrane was overlayed with rat brain lysate (1mg/ml) overnight. NudE from the lysate bound to DIC-WT and

DIC-Mut proteins was probed using NudE antibody at 1: 3000 dilution. To ensure equal loading of mDIC-WT and Mut proteins, membrane without any overlay was probed with anti-His antibody

Blot overlay assay- To detect binding of DIC-WT or Mut proteins with P150, *Dictyostelium* DIC-WT and Mut proteins were run on a non-denaturing PAGE and transferred on a PVDF membrane. Membrane was overlaid with 1mg/ml *Dictyostelium* lysate for 2 hours. To detect Dynactin P150 binding, blot was probed with anti-P150 antibody. To ensure equal His tagged DIC-WT and Mut proteins were taken for experiment, another SDS PAGE with equal volumes of both proteins was run and without any overlay the blot was probed with anti-His antibody. Similarly, for mammalian proteins, Dynactin sub-unit CC1 was purified as described above. CC1 was run on a non-denaturing gel and the membranes were overlaid with 0.5mg/ml of mDIC-WT or mDIC-Mut. To detect binding of mammalian proteins to CC1, the blot was probed with anti-His antibody.

Pulldown assays- To show interaction of His tagged DIC-WT protein with purified CC1 and P150 dynactin, 20µg of purified DIC-WT was bound to BSA blocked Ni-NTA beads for 1 hour at 4 °C. DIC-WT on beads was further incubated with either 20 µg of pure CC1 or 1mg of freshly prepared *Dictyostelium* cytosol for 3 hours. Beads were washed three times with PBS containing 0.3M NaCl to remove non-specific proteins and final elution was performed in 1X SDS reducing dye.

Bioinformatics analysis for *Dictyostelium* DIC- To determine the serine residue orthologous to S84 in rat, the dynein intermediate chain (DIC) sequences from *Dictyostelium* (XP_640973.1) and Rat (NP_001257553.1) were aligned to one another using MUSCLE 3.8 with default parameters (8). Pairwise alignment established S79 in *Dictyostelium* DIC to be the equivalent of the Rat DIC S84. To increase our confidence in the identification of the phosphorylated serine, we constructed a multiple sequence alignment of homologues of *Dictyostelium* DIC. This multiple sequence alignment was obtained by running 4 iterations of PSI-BLAST against a non-redundant protein sequence database with an *e*-value cut-off of 0.001. An analysis of the multiple sequence alignment (9) showed that the putative phosphorylatable serine was conserved in 374 of the 500 sequences (>70%).

Data representation, analysis and Statistical Methods

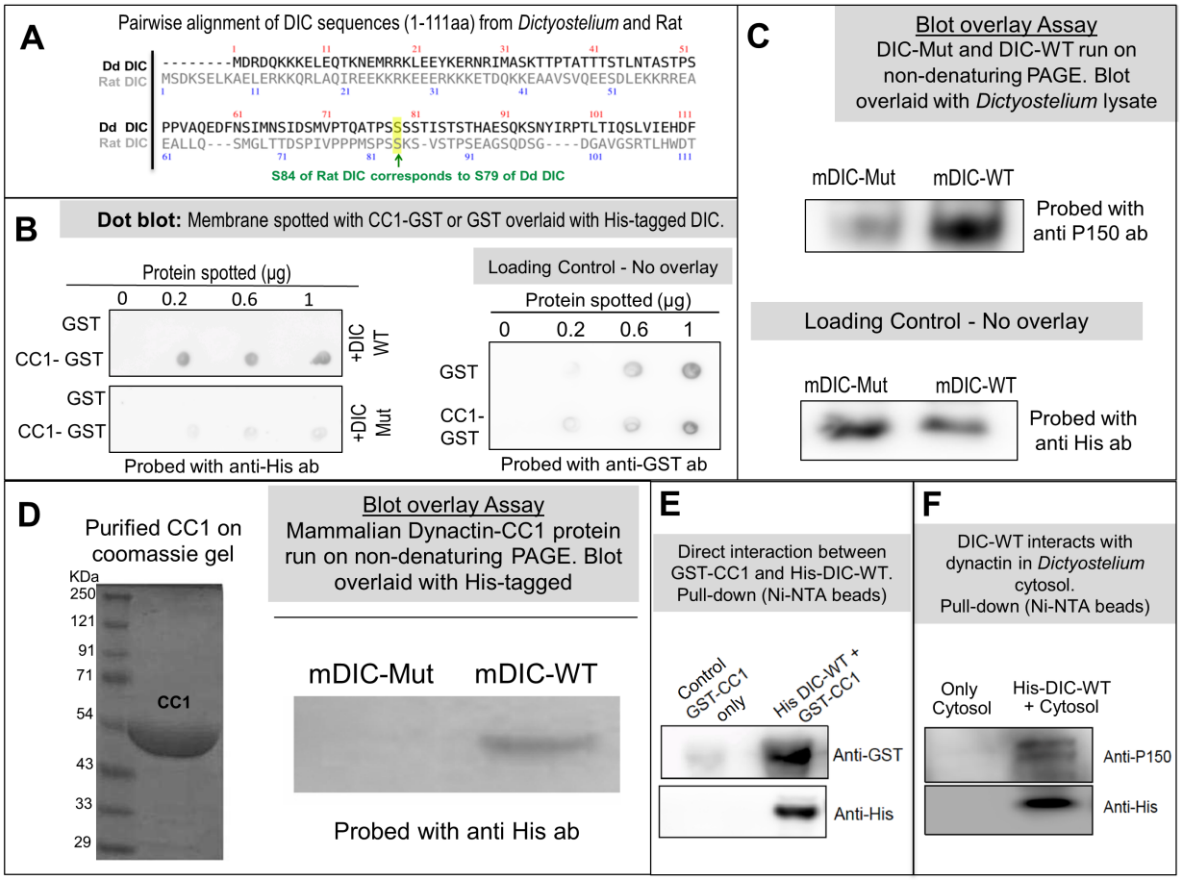
Unless otherwise stated, error bars denote the standard error of mean (SEM). Where data were known or assumed to be normally distributed, Student's t-test was used to test significance (two-tailed test, 95% confidence with null hypothesis that distributions are same). Statistical inferences for Run length, F_{STALL} and T_{STALL} were based on non-parametric tests because these quantities are known not to be normally distributed (see main text). Three different tests were employed for each case, as described in the main text:-

Bootstrapping:- We implemented a bootstrap algorithm in the "R" programming language to check whether two datasets (e.g. for Run length, or for F_{STALL}, or for T_{STALL}) were significantly different. Bootstrapping does not assume any underlying distribution of the datasets to be compared (10). A set of samples from a parent distribution is picked with replacement, the relevant statistic (e.g. the mean) is determined for this set, and this procedure is iterated many times. As an example, 10^5 bootstrap iterations were done here for every dataset. This procedure yields two sampling distributions of means (one for each dataset). Each sampling distribution is normally distributed by virtue of the Central limit theorem. The standard deviation of each sampling distribution was calculated to estimate 95% confidence intervals for the mean. The difference in these distributions was then calculated, and this difference-distribution of means was then offset to obtain a Zero-mean normal distribution of the difference in means, as described elsewhere (11). The fraction of values in this zero-mean difference distribution that exceed the difference in means between actual data sets was then calculated. This fraction is quoted as the *P*-value (null hypothesis: the two distributions are same). This bootstrap algorithm was tested

extensively using simulated datasets that were normally distributed or non-normal (e.g. exponentially decaying and containing multiple peaks).

Kolmogorov-Smirnov (KS) Test:- The KS test is a non-parametric test that assumes no specific underlying distribution of the datasets, but requires a continuous distribution of the same. KS test calculates the maximum distance (*D*) between cumulative distribution functions of the two datasets to infer statistical differences. We used the two-sample two-sided KS test available in the Origin-2020 (OriginLab) package to calculate P-values at 95% confidence (null hypothesis: the two cumulative distributions are same).

Mann-Whitney (MW) Test:- The MW test compares relative ranks of two groups. The *U* score denotes how many times one group is higher than the other in rank. We calculated the *P* value (95% confidence; two-tailed) in Origin2020.



SUPPLEMENTARY FIGURE 1: DIC-WT binds Dynactin P150 but DIC-Mut has no effect

A. Pairwise alignment of dynein intermediate chain (DIC) sequences from *Dictyostelium* and rat using MUSCLE 3.8 program with default parameters. A snapshot of the first 111 amino acids is depicted. Based on the alignment, Serine 79 of *Dictyostelium* DIC corresponds to Serine 84 of the rat sequence.

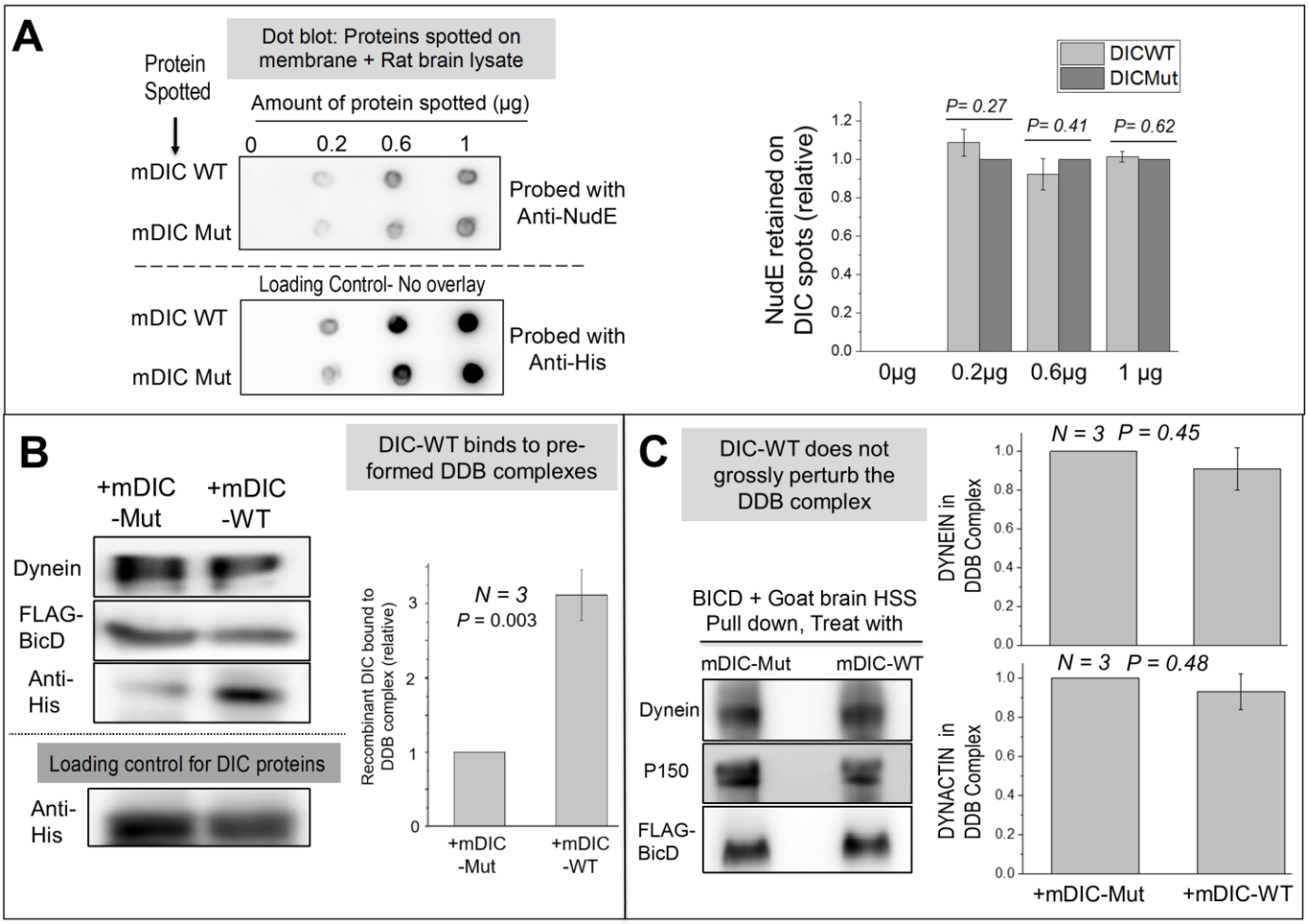
B. Dot blot showing binding of *Dictyostelium* DIC-WT and DIC-Mut proteins to *Dictyostelium* CC1 domain of dynactin (P150). DIC-WT, but not Mut showed strong binding to different amounts of CC1 protein spotted on the membrane. No non-specific binding to control GST protein was seen with either DIC-WT or DIC-Mut proteins. Right panel (Loading Control blot):- GST and CC1-GST were spotted on the membrane without any protein overlaid and directly probed with anti-GST antibody.

C. Blot overlay assay. Top - DIC-WT and DIC-Mut proteins were resolved on a non-denaturing PAGE and overlaid with *Dictyostelium* lysate. Binding of DIC proteins to P150 was detected by probing with P150 antibody. DIC-WT, but not DIC-Mut bound abundantly to P150. Lower panel (Loading control blot) :- Equal volumes of DIC-WT and Mut proteins were loaded on another SDS gel and the blot was probed with Anti-His antibody.

D. Left panel:- Coomassie gel with purified mammalian CC1 protein. Right panel:- Blot overlay assay of mammalian CC1 with mDIC-WT (S84A; mimics dephosphorylated state) or mDIC- Mut(S84D; mimics phosphorylated state). Purified CC1 was resolved on a non-denaturing PAGE and overlaid with His tagged mDIC-WT or mDIC-Mut. DIC bound to CC1 was detected by probing with anti-His antibody. The mDIC-WT did, but mDIC-Mut did not bind to CC1 in immuno-detectable range.

E. Pull-down down of GST-CC1 using His-DIC-WT bound to Ni-NTA beads. The control experiment (only GST CC1; His-DIC-WT absent) shows no signal for GST-CC1. His-DIC-WT therefore interacts directly with P150-CC1. This experiment was repeated twice with comparable results.

F. His-DIC-WT was incubated with *Dictyostelium* cytosol, followed by a pull-down using Ni-NTA beads. Endogenous *Dictyostelium* dynactin P150 was pulled down in presence of His-DIC-WT. The control experiment (Ni-NTA beads + Cytosol; No His-DIC-WT) showed no dynactin in the pull-down. This experiment was repeated thrice with comparable results.



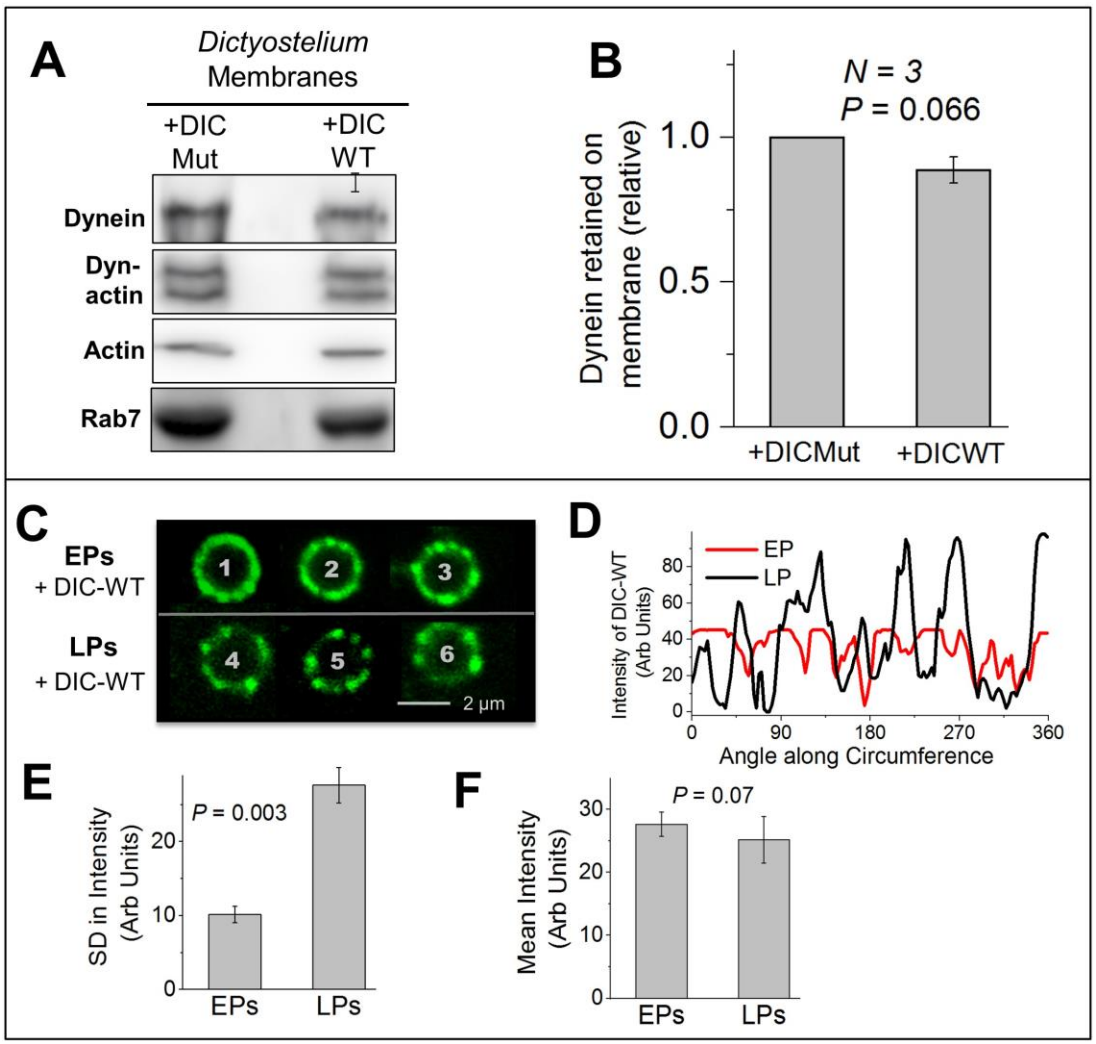
SUPPLEMENTARY FIGURE 2

The NudE-DIC interaction and Overall Integrity of Dynein-Dynactin complex are unaffected by phosphorylation state of recombinant DIC protein

A. Dot blot showing binding of mDIC-WT and mDIC-Mut to NudE. mDIC-WT and mDIC-Mut were spotted on a membrane, overlaid with rat brain cytosol, and probed with anti-NudE antibody to detect NudE binding to each protein. Lower panel (loading control):- mDIC-WT and mDIC-Mut were spotted and the membrane (without any lysate overlaid) was probed with anti-His antibody. This experiment was repeated thrice with comparable results. Right panel:- No difference was seen in binding of mDIC-WT or mDIC-Mut to NudE, suggesting that the NudE-DIC interaction is not affected by S84 phosphorylation status. *P* values obtained from Student's t-test.

B. Western blot shows enrichment of mDIC-WT (as compared to mDIC-Mut) in DDB complexes pulled down from goat brain cytosol using FLAG-BicD. Experiment was repeated three times. The mDIC-Mut band intensity was taken equal to 1 and mDIC-WT intensity calculated relative to mDIC-Mut.

C. Western blot showing levels of dynein and dynactin retained within pre-formed DDB complex. Dynein and dynactin were pulled down from goat brain lysate using recombinant FLAG-tagged BicD. The pull-down complex was then treated with mDIC-WT and mDIC-Mut proteins, and dynein/dynactin remaining bound to BicD post-treatment were assayed in the pellet. On the right is quantification showing no change in dynein or dynactin that co-precipitated with BicD. The mDIC-treatment therefore causes no gross structural change/breakage of the DDB complex. Experiment repeated three times.



SUPPLEMENTARY FIGURE 3

Effects of *Dictyostelium* DIC-WT addition.

A. Western blot showing levels of Dynein heavy chain and dynactin (P150) retained on the *Dictyostelium* membrane fraction after treatment with DIC-WT or DIC-Mut. Membranes are enriched in endosomes as seen by Rab7 band. Actin was used as loading control. The DIC-Mut band intensity was taken equal to 1 and DIC-WT intensity calculated relative to DIC-Mut.

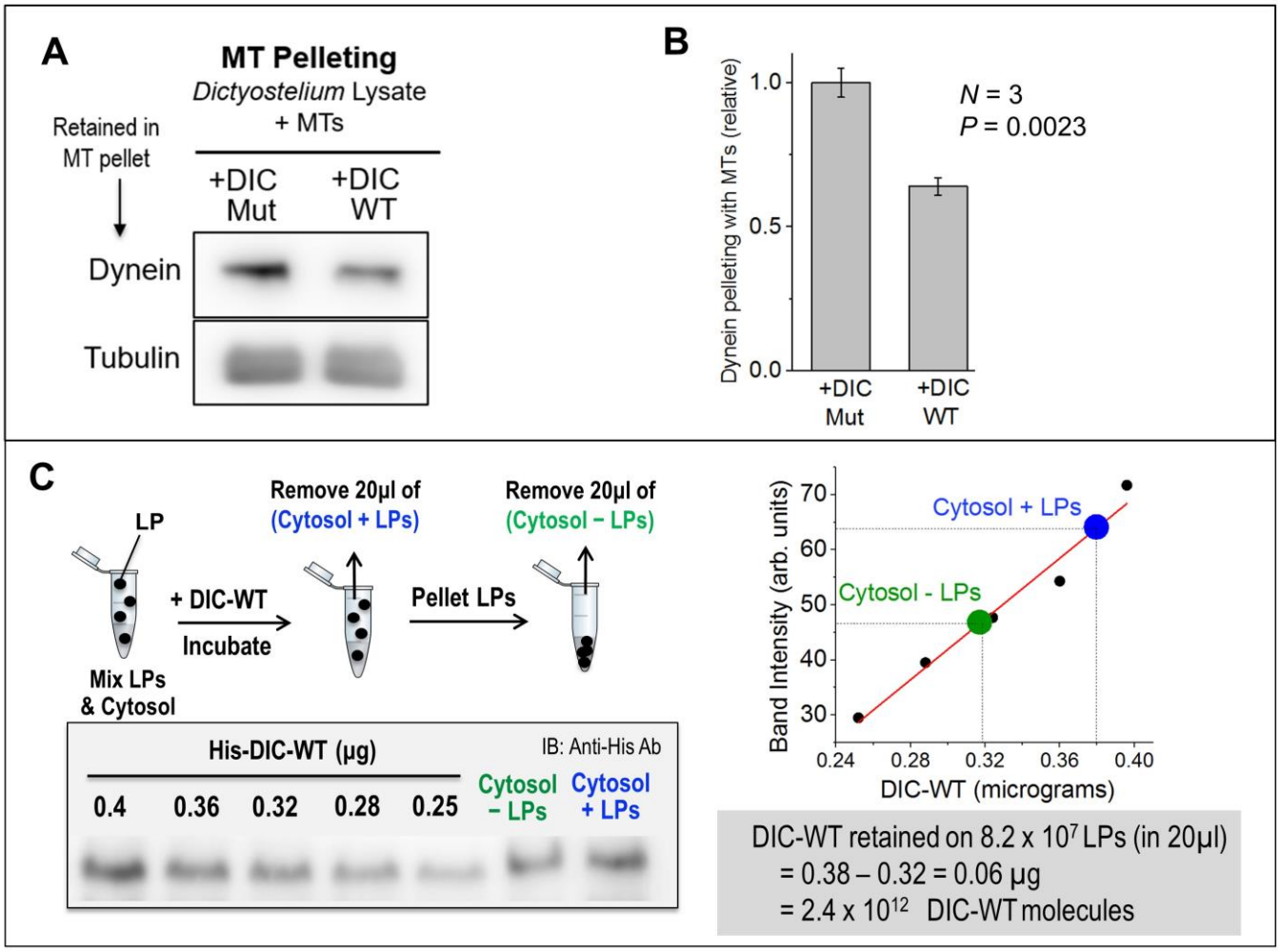
B. Quantification of (A) across three experiments. No difference in dynein levels was seen between DIC-WT and DIC-Mut treatments. Error bars, SEM. *P* values calculated using Student' t-test.

C. Confocal images showing immunofluorescence staining for DIC-WT-His for EPs (numbered 1-3) and LPs (numbered 4-6) purified from *Dictyostelium*. Bound DIC-WT was detected using Anti-His ab. The phagosomes are 2 µm in diameter. DIC-WT localization on EPs is uniform, but appears punctate on LPs mimicking the localization of endogenous dynein on LPs.

D. The pixel intensity for DIC-WT staining along circumference of an EP and an LP are plotted as a function of angle. The peak positions correspond to the puncta on LPs.

E. Fluctuation in DIC-WT staining intensity is estimated from the SD in intensity measured along the circumference. EPs show lower fluctuation (suggesting uniform staining) compared to LPs (punctate staining). Error bars represent SEM. *P* value calculated using Student's t-test.

F. The mean pixel intensity is statistically similar for EPs and LPs, suggesting no significant change in DIC-WT binding on EP versus LP. Error bars represent SEM. *P* value calculated using Student's t-test.



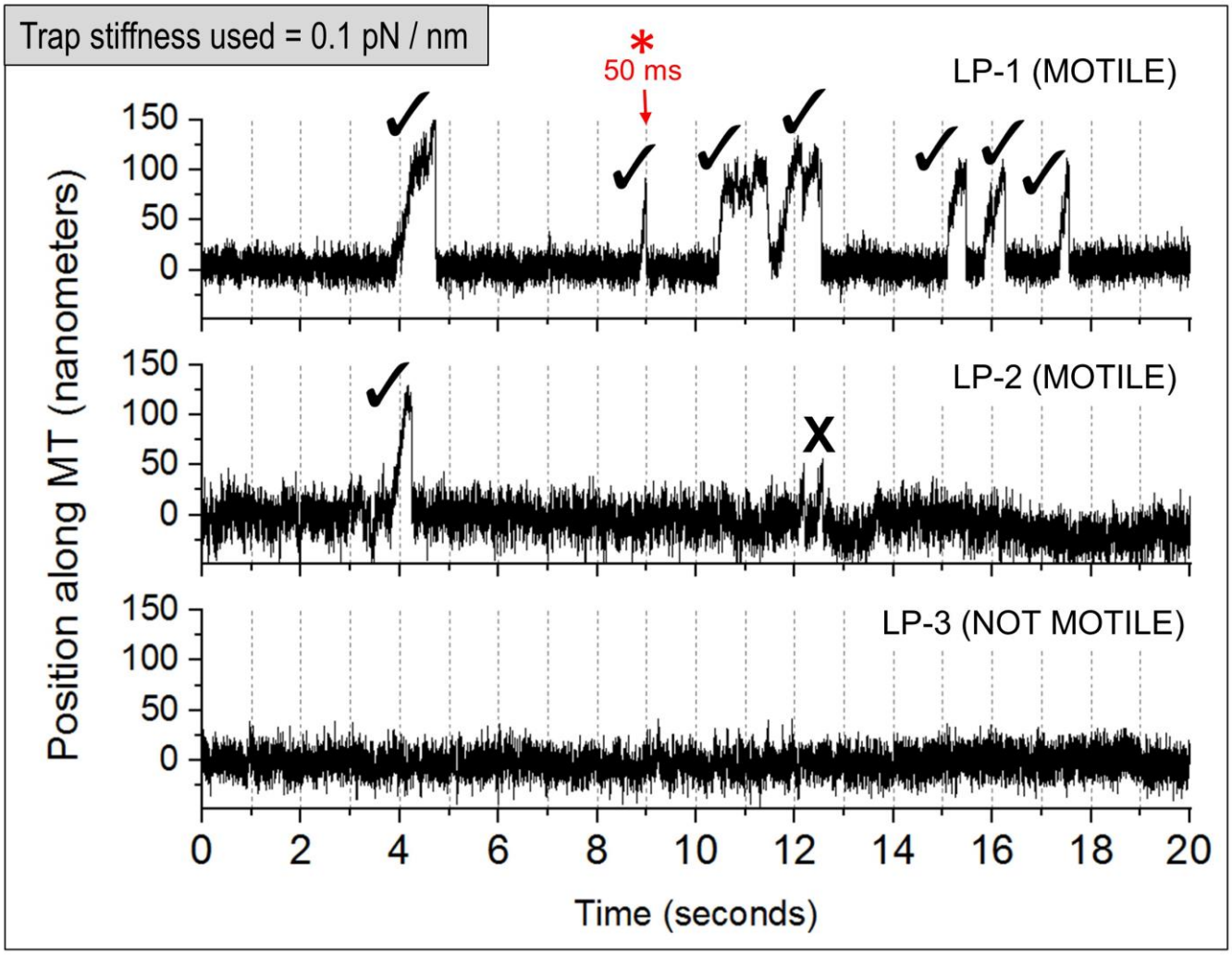
SUPPLEMENTARY FIGURE 4

MT pelleting assay in *Dictyostelium* and Optimal concentration of DIC-WT to be used in motility assays.

A. Clarified cytosol prepared from *Dictyostelium* was treated with DIC-WT or DIC-Mut, followed by addition of exogenously polymerized MTs. MTs were pelleted and Western blotting done on the pellet to estimate amount of dynein (Dhc) that co-pelleted with MTs. Tubulin from the resuspended MT pellet was used for normalization. The experiment was done after depleting ATP from cytosol. Right panel shows quantification across three independent experiments. Error bars, SEM. *P* values calculated using Student's t-test.

B. Quantification of dynein band intensities in (A). The DIC-Mut band intensity was taken equal to 1 and DIC-WT intensity calculated relative to DIC-Mut

C. Estimation of the amount of DIC-WT protein that binds to LPs. Sample was treated with DIC-WT protein and a 20µl portion was extracted [= Cytosol + LPs]. After pelleting LPs, 20µl of supernatant [= Cytosol − LPs] was extracted. The amount of DIC-WT in [Cytosol+LPs] and [Cytosol−LPs] was determined using the calibration curve (right) prepared by running known amounts of DIC-WT in a Western Blot (bottom left). The difference in DIC-WT between [Cytosol+LPs] and [Cytosol−LPs] corresponds to the DIC-WT molecules retained on LPs (calculation shown for 20µl of the sample). See main text and supplement methods for details.

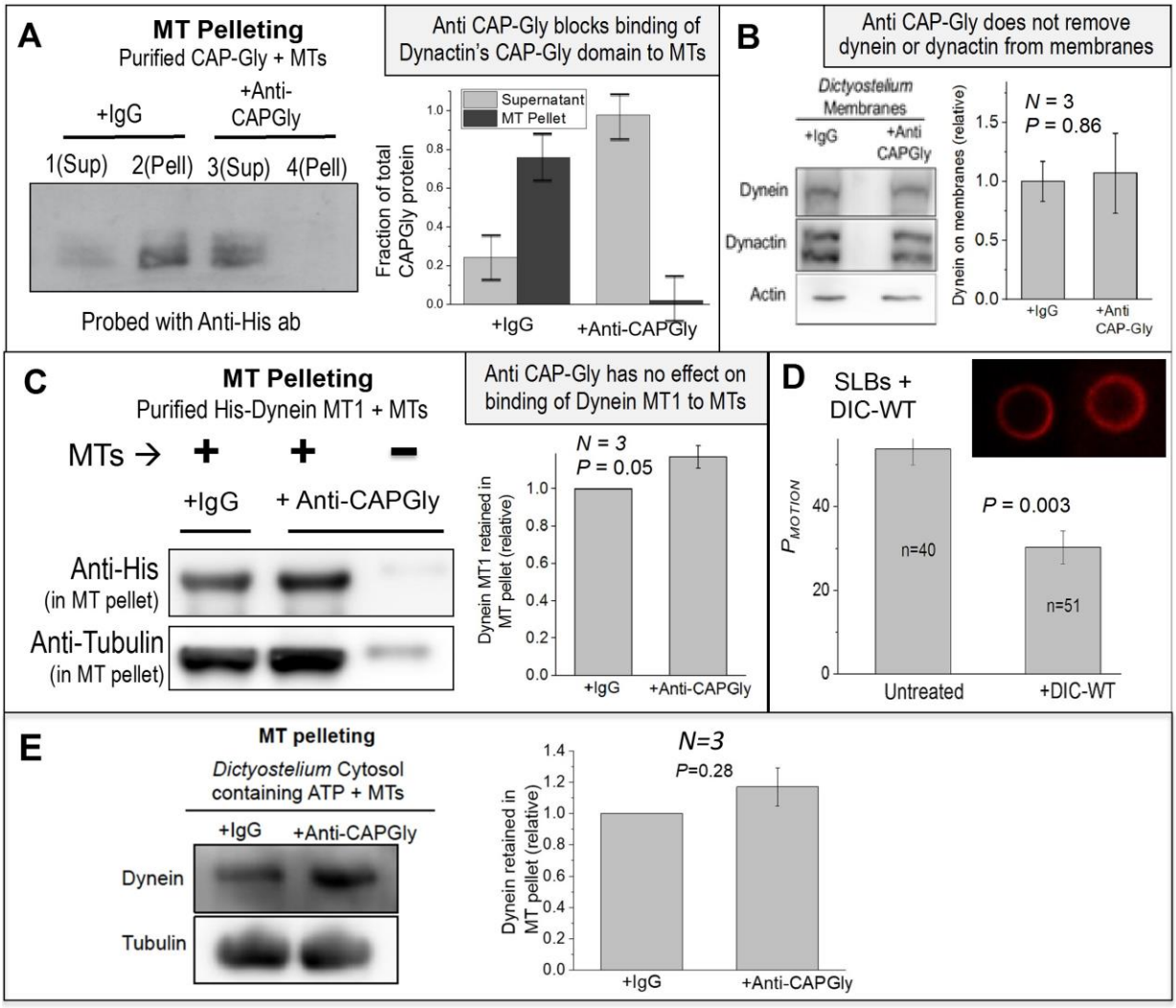


SUPPLEMENTARY FIGURE 5

Representative Optical trap records used to calculate P_{MOTION}

The displacement versus time records of three LPs held in an optical trap, as recorded in the quadrant photo detector are shown. Events marked with a "√" were counted as "activity" of dynein whereas events marked with "×" were not counted. According to our criteria (see main text), LP-1 and LP-2 were counted as "Motile", but LP-3 was not. The red star in LP-1 shows one example of a fast transient event (lasting ~50 milliseconds) that was counted as "activity". Such events would go undetected in imaging based assays.

Once attached, single dynein generates force for ~200 milliseconds (=T_{STALL}) against a load force of 1pN (see Ref. [7] in main manuscript). Therefore, K_{OFF} = 5 sec^{-1} at 1pN load. We are sampling cargo position at 1 KHz (once every millisecond) in the quadrant detector. At this resolution, force generation events lasting 20 milliseconds or more can be detected easily (for example, see LP-1 above). Even if K_{OFF} increases by 10-fold (T_{STALL} becomes 20 milliseconds), such events would still be detected. In other words, we are unlikely to miss any transient force-generation event. We therefore believe that an overwhelming majority of dynein force generation events are being detected in our assay, and P_{MOTION} obtained in this manner provides a good estimate of K_{ON}.



SUPPLEMENTARY FIGURE 6 : Effects of anti-CAPGly on Dynein/Dynactin binding to MTs and Vesicles

A. His-CAPGly domain of P150 from *Dictyostelium* was purified and treated with 0.5µM IgG or anti-CAPGly followed by addition of exogenously polymerized MTs. MTs were pelleted and amount of CAPGly protein co-pelleted with MTs or remaining in supernatant was assayed using His antibody. Anti-CAPGly antibody prevented His-CAPGly from binding to MTs, causing most of the protein to be retained in the supernatant fraction.

B. Western blot showing levels of dynein (Dhc) and dynactin (P150) retained on the *Dictyostelium* membrane fraction after treatment with either IgG or Anti CAPGly ab. Actin was used as the loading control. No difference in dynein levels was seen upon Anti-CAPGly addition. *P* values calculated using Student's t-test.

C. The microtubule binding domain of *Dictyostelium* dynein (MT1; recombinantly produced) was treated with 0.5µM IgG or anti-CAPGly, followed by addition of exogenously polymerized MTs. MTs were pelleted and amount of dynein MT1 co-pelleted with MTs was assayed. Western blot shows similar amounts of MT1 retained in the pellet fraction of both IgG and anti-CAPGly treated samples. Experiment repeated thrice with comparable results. Quantification is shown on the right. *P* values are calculated using Student's t-test.

D. **Inset** shows two Supported Lipid bilayers (SLBs) doped with 0.5 mole % rhodamine PE for visualization. Beads of 3 micron diameter were used to prepare SLBs for fluorescent imaging. **Lower panel** shows the effect of DIC-WT on SLB motion. Beads of 500nm diameter were used to prepare SLBs for motility assay. SLBs were treated with DIC-WT or left untreated. Individual SLBs were held over a MT in the optical trap for 20 secs and excursions against the trap were scored to calculate the probability of motion (P_{MOTION}). DIC-WT significantly reduced P_{MOTION}, agreeing

with the disruption of D-D interaction leading to reduced K_{ON}. This result also shows that intact dynein-dynactin complexes recruited to the SLB were driving their motion.

E. High speed supernatant from *Dictyostelium* was treated with 0.5µM IgG or anti-CAPGly in presence of ATP. This was followed by addition of exogenously polymerized MTs, incubation and centrifugation to pellet MTs. Western blot shows dynein (DHC) in the MT pellet. Tubulin from the resuspended MT pellet was used for normalization. Right panel shows quantification across three repeats of the experiment. IgG band intensity was always taken equal to 1 and Anti-CAPGly intensity was calculated relative to IgG. Error bars, SEM. P values calculated using Student's t-test.

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