Many proteins are known to promote ciliogenesis, but mechanisms that promote primary cilia disassembly before mitosis are largely unknown. Here we identify a mechanism that favours cilium disassembly and maintains the disassembled state. We show that co-localization of the S/G2 phase kinase, Nek2 and Kif24 triggers Kif24 phosphorylation, inhibiting cilia formation. We show that Kif24, a microtubule depolymerizing kinesin, is phosphorylated by Nek2, which stimulates its activity and prevents the outgrowth of cilia in proliferating cells, independent of Aurora A and HDAC6. Our data also suggest that cilium assembly and disassembly are in dynamic equilibrium, but Nek2 and Kif24 can shift the balance toward disassembly. Further, Nek2 and Kif24 are overexpressed in breast cancer cells, and ablation of these proteins restores ciliation in these cells, thereby reducing proliferation. Thus, Kif24 is a physiological substrate of Nek2, which regulates cilia disassembly through a concerted mechanism involving Kif24-mediated microtubule depolymerization.
Most quiescent and differentiated mammalian cells assemble a primary cilium, a microtubule-based projection from the cell surface. The cilium serves as a cellular ‘antenna’ for sensing and responding to the extracellular environment. Primary cilia are formed in quiescent cells, and they are resorbed as cells receive mitogenic signals1-4. Ciliary disassembly provoked by growth factor stimulation involves the activation of histone deacetylase 6 (HDAC6) at the axoneme through the concerted action of human enhancer of filaments 1 and the Aurora A kinase5-7. Deacetylation of axonemal microtubules results in destabilization of axonemal microtubules, facilitating ciliary retraction, and inhibition of Aurora A or HDAC6 blocks serum-induced ciliary resorption8.

Defects in the primary cilium have been shown to cause a spectrum of diseases, including developmental defects, obesity and polycystic kidney disease, which are collectively recognized as ciliopathies9. Defects in primary cilium assembly are also implicated in tumorigenesis, since loss of cilia is commonly associated with multiple types of cancer, including breast, pancreatic and prostatic tumours6–10. Recently, it was shown that mammary epithelial cells lose primary cilia as they undergo oncogenic transformation8,10. Breast cancers are classified into several subtypes based on gene expression profiles11. The basal subtype, which is not detectable in G1, increases in S phase and peaks in G2 phase, when it plays an established role in regulating centrosome separation12-19. Nek2 has also been implicated in suppression of primary cilium formation20. We employed a panel of centriolar markers to more precisely define the localization pattern of Nek2 within the cilium throughout the cell cycle in RPE1 cells. We found that Nek2 had two distinct patterns of localization. First, during G1 phase of the cell cycle, Nek2 could be distinguished as a centriole-bound, microtubule-depolymerizing kinesin that suppresses primary cilia formation21, but its regulation was not well understood. We find that Nek2 stably interacts with, and phosphorylates Kif24, stimulating its microtubule-depolymerizing activity. We also provide evidence that Nek2-dependent phosphorylation induces a conformational change in Kif24 that promotes its activity. Importantly, we show that Nek2–Kif24 plays a role temporally distinct from the well-established Aurora A-HDAC6 ciliary disassembly pathway by blocking the growth of new cilia and nucleation of this structure from basal bodies that have resorbed their cilia. Finally, we found that depletion of either Nek2 or Kif24 in breast cancer cell lines restored ciliation and reduced proliferation of these cells. Our results suggest the potential to target these enzymes in tumour cells.

Results
Nek2 binds and phosphorylates Kif24. To understand the molecular mechanisms involved in the regulation of primary cilium assembly and disassembly, we employed an unbiased proteomic screen and published data to identify interacting partners of Kif24 (ref. 22). This strategy allowed us to identify Nek2, a serine/threonine kinase previously implicated in centrosome separation and ciliary disassembly17,19,20. We confirmed the interaction between these proteins by co-immunoprecipitation of green fluorescent protein (GFP)-tagged Nek2 (GFP–Nek2) and flag-tagged Kif24 (f–Kif24) from transiently transfected cells and by reciprocal co-immunoprecipitation of endogenous Nek2 and Kif24 (Fig. 1a,b). We generated an array of Kif24 deletion mutants and examined interactions with Nek2, which allowed us to demonstrate that Kif24 interacts with Nek2 through two regions encompassing residues 509–547 and 924–999 (Supplementary Fig. 1).

Next we determined whether Kif24 is a substrate for Nek2. A slower migrating Kif24 species was observed when co-expressed with an active, wild-type (WT) form of Nek2 (Nek2 WT), but not with a catalytically inactive (KD) mutant (Nek2–KD; Fig. 1b). Moreover, treatment of Kif24 with λ phosphatase after co-immunoprecipitation resulted in loss of this slower migrating species (Fig. 1b), confirming that Kif24 was directly phosphorylated by Nek2 (Fig. 1c). Fragments containing residues 548–825 were most robustly phosphorylated, with an efficiency comparable to casein, a canonical substrate of Nek2 (Fig. 1c and Supplementary Figs 1D and 2). However, fragments encompassing regions spanning from the N-terminus to the catalytic motor domain (1–447) were not overtly phosphorylated, suggesting that phospho-dependent control of Kif24 is restricted to regions carboxy-terminal to the kinesin domain (548–825) that are flanked by dedicated Nek2-binding surfaces (Fig. 1c and Supplementary Fig. 1D).

Cell cycle-dependent co-localization of Nek2 and Kif24. Nek2 has been extensively studied in the context of centrosome separation during mitosis19,23. In these studies, Nek2 was reported to localize to the proximal ends of both mother and daughter centrioles19. Nek2 has also been shown to localize in a cell cycle-dependent manner to the sub-distal and/or distal ends of the centrosome and basal body, where it has been implicated in primary cilium disassembly20. We employed a panel of centriolar markers to more precisely define the localization pattern of Nek2 within the cilium throughout the cell cycle in RPE1 cells. We found that Nek2 had two distinct patterns of localization. First, during G1 phase of the cell cycle, Nek2 could be distinguished as two foci separable from centrosome distal markers, including Cep164, centrin-2, IFT88, Tel1P3 and Kif24, and it overlapped with glutamylated tubulin (GT335) and Centrosomal P4.1-associated protein (CPAP), which marks the proximal region of centrioles, and γ-tubulin at the pericentriolar matrix (Fig. 2a,c). However, during S/G2 phases, we observed an additional (third) dot of Nek2 apart from the two foci localized near the proximal ends of centrioles (marked by GT335, CPAP and γ-tubulin; Fig. 2b,c). We found that this third dot preferentially localized to the mother cilium, as demonstrated by its overlap with Cep164 and IFT88 (Fig. 2b,c). The localization pattern, proximal to Cep164 but overlapping with IFT88, centrin-2, Tel1P3 and Kif24, further indicated that Nek2 is concentrated near the distal
and Kif24 physically interact and co-localize during a portion of (Fig. 2e, ciliated panel). This supports our observation that Nek2 overlapping in time points, but localization of both proteins was largely non-
(Fig. 2e, and Supplementary Fig. 3C,D). We also found a fraction between Nek2 and Kif24 at the distal end of the mother centriole stimulation (S and G2/M phases, respectively) showed an overlap via microtubule depolymerization21. Therefore, we sought to test previous studies demonstrated that Kif24 regulates ciliogenesis Nek2-mediated phosphorylation of Kif24 blocks ciliation to be a physiological substrate of this kinase.

Nek2 expression mirrored that of Nek2 (Supplementary Fig. 3C)21. Kif24 following serum deprivation and gradually re-appeared after synchronization studies revealed that the additional Nek2 dot co-localized with Kif24 and was prevalent during the S/G2 phases (Fig. 3c and Supplementary Fig. 4C). We conclude that Nek2 co-localizes with Kif24 principally during S/G2 phases and that Kif24 is likely to be a physiological substrate of this kinase.

Nek2-mediated phosphorylation of Kif24 blocks ciliation. Our previous studies demonstrated that Kif24 regulates ciliogenesis via microtubule depolymerization21. Therefore, we sought to test functional links between Nek2 phosphorylation and ciliogenesis. Primary ciliogenesis and disassembly can be recapitulated in vitro using retinal pigment epithelial (RPE1) cells, wherein 48 h of serum starvation leads to robust ciliation, and re-stimulation of cells with serum-containing media (termed serum re-stimulation) leads to ciliogenesis (Supplementary Fig. 3B)3. We found that Nek2 expression steadily declined following serum deprivation and gradually re-appeared after 18–24 h of serum stimulation (Supplementary Fig. 3C). Kif24 expression mirrored that of Nek2 (Supplementary Fig. 3C)21. Most of the cells lacking cilia at 18 and 24 h post-serum re-stimulation (S and G2/M phases, respectively) showed an overlap between Nek2 and Kif24 at the distal end of the mother centriole (Fig. 2e, and Supplementary Fig. 3C,D). We also found a fraction of ciliated cells that expressed both Nek2 and Kif24 during these time points, but localization of both proteins was largely non-overlapping in ~76–86% of these re-stimulated, ciliated cells (Fig. 2e, ciliated panel). This supports our observation that Nek2 and Kif24 physically interact and co-localize during a portion of

Figure 1 | Nek2 stably interacts with, and phosphorylates, Kif24. (a) Lysates of HEK293 cells were immunoprecipitated and immuno-blotted with the indicated antibodies to demonstrate reciprocal interactions between Nek2 and Kif24. IgG indicates negative control derived from rabbit (Left panel) or mouse (Right panel). Input and immunoprecipitated samples were electrophoresed on the same gels. (b) The indicated Flag- and GFP-tagged proteins were expressed in HEK293 cells and immunoprecipitated, after which they were treated with λ-phosphatase or left untreated, and immuno-blotted with anti-Flag and GFP antibodies as indicated. (c) In vitro kinase assays were performed with the indicated fragments of Kif24 as GST fusions and purified Flag-tagged WT or KD Nek2. Uncropped western blots are shown in Supplementary Fig. 13.
in vitro kinesin motif alone was able to depolymerize microtubules (KEC) form, and (2) a purified fragment of Kif24 containing the kinesin motif by showing that (1) abnormally long centrioles and primary cilia formation through its Nek2 directly stimulates Kif24 activity by phosphorylation of new cilia. Likely acts to prevent assembly of nascent cilia and the nucleation of new cilia.

Nek2 directly stimulates Kif24 activity by phosphorylation. We previously reported that Kif24 suppresses the aberrant growth of centriolar microtubules and primary cilia formation through its kinesin motif by showing that (1) abnormally long centrioles generated by Cep97 depletion in U2OS cells were abolished by expression of active Kif24, but not by a catalytically inactive (KEC) form, and (2) a purified fragment of Kif24 containing the kinesin motif alone was able to depolymerize microtubules in vitro. Therefore, we tested whether Nek2 could regulate the microtubule-depolymerizing activity of Kif24 using analogous approaches. First, we found that depletion of Nek2 had minimal impact on centriolar elongation in U2OS cells, irrespective of expression of active or inactive Kif24 (Fig. 3d). As expected, Cep97 ablation led to aberrant elongation of centrioles in controls and cells expressing inactive Kif24, whereas expression of active Kif24 suppressed this phenotype (Fig. 3d and Supplementary Fig. 7A). Strikingly, however, ectopic expression of Kif24 did not rescue the centriolar phenotype in cells that were co-depleted of Nek2 and Cep97 (Fig. 3d).

Next, we tested the impact of Nek2-mediated phosphorylation of Kif24 on microtubule-depolymerization in vitro. Our attempts at expressing full-length Kif24 were unsuccessful, owing to the presence of an amino-terminal domain that destabilizes the protein. Therefore, we expressed a flag-tagged Kif24 fragment lacking the first 92 amino acids (93–1,368) in insect cells, with or without Nek2 (Fig. 3e). We purified this recombinant Kif24, and in parallel, we treated protein obtained from cells that co-expressed Nek2 with λ-phosphatase, which resulted in an increase in electrophoretic mobility, indicating that Kif24 was efficiently dephosphorylated (Fig. 3e). Next, we investigated whether Nek2-mediated phosphorylation could augment the microtubule-depolymerizing activity of Kif24. In agreement with our in vivo analysis, we observed a significant (>2 fold) increase in microtubule depolymerization when Kif24 was co-expressed with Nek2, as compared with Kif24 alone. However, this increase was eliminated with λ-phosphatase treatment. Moreover, we examined a recombinant fragment of Kif24 encompassing the kinesin domain that lacks putative Nek2 phosphorylation sites (Kif24 (93–547); Fig. 1c) and found that its activity was not augmented by Nek2 (Supplementary Fig. 7B). These data collectively suggest that Nek2-mediated phosphorylation of
showed dominant negative effects on ciliation, similar to the KEC 621–638 (Kif24 deletion mutants, encompassing residues 603–622 or ciliation (Supplementary Fig. 5A). Notably, we found that certain of Kif24 internal deletion mutants and tested their impact on regions spanning 548–678 and 671–825 (Fig. 1c). To delimit the Fig. 5B). Nek2 potently phosphorylates Kif24 residues within the significantly increasing ciliation (Fig. 4c and Supplementary (Fig. 4b,e and f, and Supplementary Fig. 5D,E). In growing RPE1 cells were treated with the indicated siRNAs and transfected to overexpress Flag-tagged Kif24 (f–Kif24) or f–Nek2 as shown. (d) U2OS cells were depleted of Cep97 and/or Nek2 with ectopic expression of f–Kif24, or f–KEC Kif24, as indicated, and the per cent of cells with elongated centrioles was measured. (e) (Left) In vitro microtubule-depolymerization assays were performed with polymerized tubulins and the indicated purified recombinant proteins, which were either treated with λ phosphatase or left untreated. The reaction products were separated into supernatant (s) and pellet (p) fractions to measure release of free tubulins. The migration of phosphorylated and unphosphorylated Kif24 and tubulins is shown. (Right) Quantitation of data shown at left. Tubulin alone was used as control. Fold increase of Tubulin S/(P+S) per sample is normalized to the Tubulin alone control. Data were obtained from three biologically independent experiments. Error bars show s.e.m. *P<0.05, **P<0.001. Statistical significance was tested by comparisons with NS siRNA (a) or f–Kif24 (d), or as indicated by bars in b, c and e. Uncropped western blots are shown in Supplementary Fig. 13. EV, empty vector control.

Kif24 facilitates its microtubule-depolymerizing activity in vivo and in vitro, thereby regulating ciliogenesis.

We next set out to identify the Nek2 phosphorylation site(s) that regulate Kif24 activity by systematically mutagenizing Kif24 and testing the impact of these mutants on ciliogenesis. We examined cilium assembly in RPE1 cells expressing active (WT) or inactive (KEC) Kif24, as well as mutants that abrogated Kif24 phosphorylation (Fig. 4a and Supplementary Fig. 5A). We also expressed each protein in cells that were engineered to stably express active (WT) or inactive (KD) Nek2 and were serum starved for 48 h to determine their ability to suppress ciliation (Fig. 4b,e and f, and Supplementary Fig. 5D,E). In growing RPE1 cells, expression of KEC-Kif24 behaved as a dominant negative by significantly increasing ciliation (Fig. 4c and Supplementary Fig. 5B). Nek2 potently phosphorylates Kif24 residues within the regions spanning 548–678 and 671–825 (Fig. 1c). To delimit the residues required to stimulate Kif24 activity, we expressed a series of Kif24 internal deletion mutants and tested their impact on ciliation (Supplementary Fig. 5A). Notably, we found that certain Kif24 deletion mutants, encompassing residues 603–622 or 621–638 (Δ603–622, Δ621–638), but not others (Δ595–613), showed dominant negative effects on ciliation, similar to the KEC mutant, suggesting that a region spanning residues 603–638 contains a key regulatory phosphorylation site(s) responsible for Kif24 activation (Supplementary Fig. 5B). Next, we performed the converse experiment, examining whether the increase in ciliation provoked by Kif24 deletion in growing RPE1 cells can be rescued by expression of RNAi-resistant Kif24 mutants. We found that Kif24 (Δ595–613) rescued the depletion phenotype, whereas the activity of Kif24 (Δ621–638) and Kif24 (Δ603–622) was significantly compromised (Supplementary Fig. 5C). Furthermore, co-expression of Kif24 (Δ595–613) and WT-Nek2 in cells that were subsequently starved for 48 h resulted in a substantial decrease in ciliation, similar to expression of WT-Nek2 and Kif24, in sharp contrast with cells expressing WT-Nek2 alone, or WT-Nek2 and KEC-Kif24 (Supplementary Fig. 5D). Co-expression of KD-Nek2, on the other hand, abolished the impact of all active Kif24 deletion mutants on ciliation (Supplementary Fig. 5E), further confirming that Nek2 is indispensable for Kif24 function.

The internal deletions (Δ603–622 and Δ621–638) shared two overlapping, potential phospho-sites (T621/S622; Supplementary Fig. 5A), suggesting that these residues constitute regulatory targets of Nek2. Therefore, we generated a double point-mutated form of Kif24 (T621A/S622A, TS-A) in the GST–Kif24 fragment spanning from 585–645 and found that by in vitro kinase assay,
the mutant exhibited reduced phosphorylation by Nek2 as compared with the WT fragment (Supplementary Fig. 8A). Next, we mutagenized full-length Kif24 to further determine whether these residues are required for Kif24 regulation. Using WT Kif24 and Kif24 (KEC) as positive and negative controls, respectively, we determined whether Kif24 (TS-A) has a dominant negative effect by altering ciliary induction in growing cells with or without depletion of Kif24 (Fig. 4c,d) and whether it can impact ciliation in the presence of active or inactive Nek2 in serum-starved cells (Fig. 4e,f). Conversely, we also created phospho-mimetic mutants in which we converted both residues to aspartic acid (T621D/S622D). We found that these mutations phospho-mimetic mutants in which we converted both residues to aspartic acid (T621D/S622D). We found that these mutations did not mimic the phosphorylated state, as they did not result in constitutive activation of Kif24 in the absence of Nek2, and therefore, we did not further pursue the use of this mutant (Supplementary Fig. 9). Remarkably, however, we found that the S789A mutation led to a sharp decline in signal, suggesting that this residue is phosphorylated by Nek2 (Supplementary Fig. 8B). We found that the S789A mutation did not significantly alter the extent of ciliation when combined with Δ595–638 (Supplementary Fig. 8C), further confirming that residues T621 and S622 of Kif24 constitute specific, critical regulatory targets of Nek2.

**Phosphorylation leads to conformational change of Kif24.** Kif24 is a member of the Kinesin-13 family, and it has been shown that phosphorylation of other family members alters their activity. Therefore, we did not further pursue the use of this mutant (Supplementary Fig. 9). Remarkably, however, we found that Kif24 (TS-A) largely nullified Kif24 activity in each of these assays, even in the presence of exogenous WT-Nek2 (Fig. 4). Furthermore, we compared the sub-cellular localization of both WT Kif24 and Kif24 (TS-A) during the G0, G1, S and G2 phases of the cell cycle and found that they were similar, suggesting that the loss of activity in the Kif24 (TS-A) mutant is not due to aberrant localization (Supplementary Fig. 10). We noted that other Kif24 residues appeared to be robustly phosphorylated by Nek2 in vitro, including a region spanning residues 671–825 (Fig. 1c), which prompted us to create two Ser-to-Ala mutations within this fragment (S789A and S810A). We found that the S789A mutation led to a sharp decline in signal, suggesting that this residue is phosphorylated by Nek2 (Supplementary Fig. 8B). However, the S789A mutation did not significantly alter the extent of ciliation when combined with Δ595–638 (Supplementary Fig. 8C), further confirming that residues T621 and S622 of Kif24 constitute specific, critical regulatory targets of Nek2.
change using a Kif24 Förster resonance energy transfer (FRET) (FR-Kif24) probe with an amino-terminal mCitrine acceptor fluorophore and a carboxy-terminal mCerulean donor fluorophore (Fig. 5a). The probes were expressed in HEK293 cells, and the efficiency of energy transfer between the donor and acceptor fluorophores was measured. First, as expected, we observed low FRET in cells that co-expressed soluble mCerulean and mCitrine, whereas tethering of mCitrine to mCerulean produced strong FRET (Fig. 5a,b). Next, we tested whether Nek2 altered the conformation of Kif24 by co-expressing the FR-Kif24 probe with functional Nek2 (WT Nek2). We observed a significant decrease in the FRET signal in cells that expressed Nek2, whereas co-expression of FR-Kif24 with catalytically inactive Nek2 (KD Nek2) caused no significant change in FRET (Fig. 5c). We further created a mutant version of FR-Kif24 in which sites phosphorylated by Nek2 were changed to alanine (FR-Kif24 (TS-A)). This probe exhibited similar FRET levels to the WT probe, but unlike the WT probe, co-expression of WT Nek2 did not alter the FRET signal (Fig. 5c). Furthermore, we found that the FRET of FR-Kif24 increased significantly in cells deprived of endogenous Nek2 (Supplementary Fig. 11B). Taken together with our observation that the amino- and carboxy-terminal regions of Kif24 can interact, these data suggest that Kif24 undergoes a conformational change in response to Nek2-mediated phosphorylation at T621/S622, which results in an ‘open’ conformation in which the amino- and carboxy- termini are separated (Fig. 5d). These findings are reminiscent of a ‘tail inhibition’ model, wherein the interaction between the head (containing the motor domain) and tail motifs in a cohort of myosin and kinesin motor proteins leads to an auto-inhibitory conformation that is reversed in response to activating signals29,30.

Activated Kif24 disassembles cilium during G2/M phases. Although primary cilia are known to assemble in a fraction of proliferating RPE1 cells, ciliogenesis is highly induced as cells enter quiescence, and the organelle disassembles when quiescent cells are exposed to growth factors1–4. Cilium resorption has been shown to occur in two stages: the first stage occurs immediately after serum re-stimulation, followed by a robust wave of resorption at the G2/M transition1–4. It is attractive to posit that ensuring cilium resorption during the G2/M phase constitutes an additional fail-safe barrier, as this organelle is incompatible with a mitotic spindle. Cilium disassembly is triggered by a complex mechanism involving activation of Aurora A, which stimulates HDAC6-mediated deacetylation and destabilization of axonemal microtubules, leading to cilium resorption3. Since the Nek2–Kif24 pathway negatively regulates

Figure 5 | Nek2-dependent phosphorylation induces conformational change in Kif24. (a) Schematics of constructs used for FRET assay. (b,c) FRET signals normalized to donor emissions (I_F/I_D, FRET ratio) of the indicated samples. Co-expressed mCitrine and mCerulean (mCit. – mCer.) was used as negative control and tethered mCitrine–mCerulean (mCit.–mCer.) as a positive control displaying high FRET. Expression of probes and Nek2 in panel (c) was confirmed by immunoblotting with anti-GFP antibodies to detect FRET probes (FR-Kif24 and FR-Kif24 (TS-A)) or anti-Flag antibodies to detect tagged Nek2. β-actin was used as a loading control. Data were obtained from three independent experiments. Error bars show s.e.m. *P < 0.05. (d) Model of Kif24 activation by Nek2.
First, we examined the expression profiles of Nek2 and Kif24 at various times after serum re-stimulation of quiescent cells. We found that Nek2 and Kif24 were expressed from 18 h onward, whereas they were not detectable at earlier time points that we examined (Supplementary Fig. 3C), suggesting that Nek2–Kif24 could play a role in the second ‘phase’ of ciliary disassembly during the later stages of the cell cycle. Next, we ablated Nek2 and/or Kif24 at 12 h post-serum deprivation (to avoid the consequences of eliminating Nek2 and/or Kif24 in growing cells, which would promote premature ciliation) and visualized cilia after 48 h of serum starvation (0 h time point), and 6, 18 and 24 h post-serum stimulation (Fig. 6a). We compared the kinetics of this response to cells treated with specific inhibitors of Aurora A (PHA-680632) and HDAC6 (Tubacin) at each time point (Fig. 6a). In accordance with the expression profiles of Nek2 and Kif24, depletion of Nek2 and/or Kif24 had no effect on ciliation after 6 h re-stimulation (Fig. 6b). Interestingly, however, we observed dramatic assembly of primary cilia at a later time point (24 h) in cells lacking Nek2 and/or Kif24, which likely reflects de novo cillum assembly and/or possible re-growth of cilia that may have previously commenced or completed the disassembly process (Fig. 6b). This suggests that cillum assembly and disassembly co-exist in a dynamic equilibrium in which the concerted action of Nek2 and Kif24 can prevent mother centrioles/basal bodies from nucleating a new cilium.

Further, we could distinguish the activities of Nek2–Kif24 from the Aurora A-HDAC6 pathway, since treatment of serum re-stimulated cells with either Aurora A or HDAC6 inhibitors at 0 h clearly prevented cilia disassembly as compared with the dimethylsulphoxide control (Fig. 6c). We reasoned that overlapping roles of these two pathways might prevent us from detecting any differences in ciliation if both pathways were ablated. Therefore, to distinguish the role of Nek2–Kif24 in cilia disassembly from the Aurora A-HDAC6 pathway, we depleted Kif24 and/or Nek2 from serum-starved cells and treated them with Tubacin or PHA-680632, from 6 h post-re-stimulation (Fig. 6a). In contrast with the consistent level of ciliation observed in the drug-treated, control siRNA-treated cells, an increase in cilia formation was observed in Nek2- and/or Kif24-depleted cells, which persisted throughout drug treatment at 18 and 24 h post-serum stimulation (Fig. 6d). This suggests that Nek2–Kif24 and Aurora A-HDAC6 play distinct, sequential roles during cilia disassembly as cells re-enter the cell cycle from quiescence: Aurora A-HDAC6-mediated axonemal disassembly is succeeded by Nek2–Kif24-mediated suppression of nascent cilium assembly and, potentially, ciliary re-assembly in cells that have already

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**Figure 6** The role of Kif24 can be distinguished from the Aurora A-HDAC6 pathway during primary cilium disassembly. (a) Schematic illustration of experimental strategy. (b–d) RPE1 cells were transfected with siRNAs or treated with inhibitors during the indicated periods, and ciliation was assessed at each indicated time points. Treatments with inhibitors of HDAC6 (Tubacin) and Aurora A (PHA-680632) were performed as indicated. Cells were collected at the indicated times after serum re-stimulation. Data were obtained from three biologically independent experiments. Error bars show s.e.m. *P<0.05. Statistical significance was tested against NS siRNA at 24 h (b), dimethylsulphoxide treatment at 6, 12 and 24 h (c), and at 6 h serum re-stimulated time point for each drug treatment (d).
disassembled their cilia. In this sense, Kif24 activity could ensure the completion of cilium removal in the later stages of the cell cycle.

A potential role for Nek2 and Kif24 in mammary tumorigenesis. It has recently been shown that Nek2 is a proto-oncogene that is highly expressed in various cancer types, including breast cancer13,15. Furthermore, it is known that mammary epithelial cells lose primary cilia during the process of oncogenic transformation8,10. Therefore, we compared the expression levels of Kif24 and Nek2 in a panel of breast cancer cell lines with the normal human mammary epithelial cell line (HMEC) as control. By normalizing the expression profiles of Kif24 and Nek2 with HMECs, we found that Kif24 protein levels were comparable or modestly elevated across the panel of cell lines, whereas Nek2 was strongly upregulated (~5–30-fold) in all breast cancer cell lines that we examined (Supplementary Fig. 12A–C). Although they are non-tumorigenic, MCF10A are immortal and hyperplastic, and these cells also exhibited enhanced expression of Nek2 (refs 10,11). This led us to hypothesize that in hyper-proliferative mammary and breast cancer cells, the uncontrolled phosphorylation of the basal kinase, properties reminiscent of a group of kinesin and kinesin-related kinases35,36. In future studies, it will be interesting to dissect the molecular determinants that confer such distinct properties on Kif24 and Nek2 orthologs in ciliary and flagellar assembly/disassembly.

We showed that there are two regions of Kif24 (a middle and carboxy-terminal domain) required for interaction with Nek2 (Fig. 5d and Supplementary Fig. 1). Further, our structure-function and mechanistic studies revealed that Kif24 exhibits intra-molecular interactions, which promote a ‘closed’, auto-inhibited state, properties reminiscent of a group of kinesin and myosin motor proteins29,30. Moreover, analogous to other kinesins, we found that binding by Nek2 and activating phosphorylation promotes an ‘open’ conformation of Kif24. In contrast, others have shown that Aurora B-mediated phosphorylation induces a conformational change that stabilizes the ability of Kif24 to depolymerize microtubules. Nek2 and Kif24 co-localize at the distal ends of mother centrioles in S/G2 phase, consistent with a role in ensuring the disassembly of a ciliary axoneme, which is incompatible with a mitotic spindle in mammalian cells37–40. Proteins related to Kif24 and Nek2 are found in diverse ciliated and flagellated species, including Chlamydomonas, Tetrahymena, trypanosomes and Giardia, wherein orthologs of Kif24 (Kinesin-13) and Nek2 (NIMA-related kinases) have been implicated in flagellar assembly and disassembly41–43. Intriguingly, Chlamydomonas Kinesin-13 protein (CrKinesin 13) functions in both processes, and it is targeted to the disassembling flagella and is phosphorylated as new flagella are assembled44,45. In addition, two NIMA-related kinases, FA2 and CNK2, have been shown to regulate flagella assembly and disassembly in Chlamydomonas42,46. CNK2 localizes to the axoneme, where it can modulate flagellar length by altering the rate of axoneme disassembly42. Likewise, overproduction of a Tetrahymena kinase related to CNK2 reduces cilia length43. Moreover, it has recently been reported that a member of the mammalian Kinesin-13 family, Kif2A is phosphorylated by Plk1 at the sub-distal appendage of the basal body to facilitate primary cilium disassembly shortly (within 4 h) after serum stimulation of quiescent cells47. Thus, although there appear to be both mechanistic similarities and differences across species, these findings suggest a widespread, evolutionarily conserved role for Kinesin-13 and Nek2 orthologs in ciliary and flagellar assembly/disassembly.

We showed that there are two regions of Kif24 (a middle and carboxy-terminal domain) required for interaction with Nek2 (Fig. 5d and Supplementary Fig. 1). Further, our structure-function and mechanistic studies revealed that Kif24 exhibits intra-molecular interactions, which promote a ‘closed’, auto-inhibited state, properties reminiscent of a group of kinesin and myosin motor proteins29,30. Moreover, analogous to other kinesins, we found that binding by Nek2 and activating phosphorylation promotes an ‘open’ conformation of Kif24. In contrast, others have shown that Aurora B-mediated phosphorylation induces a less active, ‘open’ conformation of Kif2C/MCAK (ref. 28), suggesting inherent differences between the members of the Kinesin-13 family. In future studies, it will be interesting to dissect the molecular determinants that confer such distinct properties on Kif2C/MCAK and Kif24.

Our Kif24 ablation data have two important implications for our understanding of cilium assembly and disassembly during the cell cycle. First, our experiments suggest that conditions are permissive to cilium assembly throughout interphase, and that elimination of certain barriers, enforced by Nek2 and its substrate, Kif24, and possibly other proteins, unveils this permissivity. Even as late as S and G2 phase, assembly and disassembly may be in equilibrium, but the equilibrium is shifted
Nek2 versus Nek2

Statistical significance was tested against NS siRNA for each cell line (as indicated) in Hs578T cells. (Kif24. Primary cilia (antibodies against glutamylated tubulin (GT335) and Arl13B, in the MCF10 cell line series used in our study. Scale bar: 5 μm). For panels (c) and (d), statistical analyses were performed by comparing samples indicated by brackets: NS versus Nek2 or Kif24 siRNA-treated cells, Nek2 versus Nek2 + Talpid3 siRNA-treated cells, and between Kif24 and Kif24 + Talpid3 siRNA-treated samples (d). Error bars show s.e.m. *P<0.05.

Figure 7 | Removal of Nek2 or Kif24 restores ciliation in breast cancer cell lines. (a) Immunofluorescence detection of primary cilia, visualized with antibodies against glutamylated tubulin (GT335) and Arl13B, in the MCF10 cell line series used in our study. Scale bar: 5 μm. (b) Depletion of Nek2 or Kif24 restores ciliation in a subset of MCF10 cell lines. (c) Proliferation (Ki-67 positivity) and ciliation were assessed in Hs578T cells depleted of Nek2 and/or Kif24. Primary cilia (d) and proliferation (Ki-67 positivity) (e) were counted after RNAi-mediated depletion of Kif24 or Nek2 in combination with Talpid3 (as indicated). (f) Summary of our findings in breast cancer cells. Data were obtained from three biologically independent experiments. Statistical significance was tested against NS siRNA for each cell line (b) and for each parameter tested (ciliation, black bar or Ki-67 positivity, white bar) (c). For panels (d) and (e), statistical analyses were performed by comparing samples indicated by brackets: NS versus Nek2 or Kif24 siRNA-treated cells, Nek2 versus Nek2 + Talpid3 siRNA-treated cells, and between Kif24 and Kif24 + Talpid3 siRNA-treated samples (d,e). Error bars show s.e.m. *P<0.05.

...towards the unciliated state through the timely expression of Nek2 and Kif24 during this period of the cell cycle. Although we favour a model in which Nek2 and Kif24 prevent re-growth of cilia that have previously resorbed as well as de novo assembly of the organelle, we have not distinguished between these possibilities in our studies, and future experiments will be required to determine whether this is indeed the case. Second, our data suggest that Kif24 function, coupled to Nek2 activity, provides a counterpart to the Aurora A-HDAC6 pathway of cilium disassembly initiated during cell cycle re-entry. An analogous mechanism may operate in Chlamydomonas, wherein the Nek2-related kinase, CNK2, supports a feedback control mechanism to maintain the balance between the assembly and disassembly of the axoneme, and the protein induces the disassembly process48. Similarly, Nek2 in mammalian cells phosphorylates Kif24 to establish a fail-safe mechanism by preventing ciliogenesis—a team-effort involving Hercules (Aurora A-HDAC6) and Iolaus (Nek2–Kif24) may be required to ensure the loss of cilia before mitosis.

Our studies on Kif24 and Nek2 also provide potentially useful clinical avenues of exploration. Nek2 was recently identified as an oncogene, and its expression was found to be upregulated in numerous tumour types, including breast tumours13–15. For example, a approximately ninefold increase in the per cent of cells with 'strong' Nek2 protein staining was observed in tumours, as compared with normal breast tissue, and its upregulation was closely associated with poor prognosis and high recurrence rates in patients13,14. These findings are consistent with our observation that Nek2 proteins levels are highly upregulated in breast cancer cell lines (Supplementary Fig. 12A–C)13–15. Our data implicate Kif24 as a physiological substrate of Nek2, among the few that have been identified19,49–51. While Nek2 is likely to have many substrates relevant to growth control, we have identified Kif24 as an important target, and our studies suggest that either could be rate-limiting for proliferation in certain circumstances. Both Kif24 and Nek2 are enzymes, suggesting that they are potentially druggable. Indeed, progress has been made in developing inhibitors of Nek2 (ref. 52). Our data suggest the utility of inhibiting their function in human tumours. Importantly, our experiments with the MCF10 cell line model suggest a means for therapeutic intervention, although our results strongly imply that such intervention must occur in the early stages of progression of the tumour to ensure efficacy. Clearly, tumours in the early stages of the disease retain the capacity to ciliate5, and strategies directed at these populations are therefore more likely to succeed. Future studies in other tumour types will be required to explore the generality of this therapeutic strategy.
Methods

Cell culture and plasmids. Human RPE1-hTERT, U2OS and HEK293 cells were obtained from the American Type Culture Collection and grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The human breast cancer cell lines MCF7, ZR57-1, MDA-MB-231, MDA-MB-468, HS578T and MCF10A were obtained from American Type Culture Collection, and the LLCMC cell line was obtained from Lonza. The cell lines engineered to express H-ras, H-ras+ and MCF10A-CAT13 were obtained from Karmanos Cancer Institute, and MCF10DCIS.com was obtained from Wayne State University. HMEC cells were cultured in MEGM SingleQuot Kit Supplement & Growth Factors. MCF7, MDA-MB-231, MDA-MB-468, HS578T, MCF10DCIS.com and MCF10A-C11 were maintained in DEMEM supplemented with 10% FBS, MCF10A and MCF10A1T cell lines were maintained in DEMEM/F-12 supplemented with 0.1 μg ml−1 cholera toxin, 10 μg ml−1 insulin, 0.5 μg ml−1 hydrocortisone, 0.02 μg ml−1 epidermal growth factor (EGF) and 5% horse serum. The MCF10A, AT1, DCS.com and CA1 cell lines were cultured for 6 days in growth medium to reach confluence, before the induction of cilia. SF9 and High Five cells were grown in Grace’s insect medium supplemented with 10% FBS at 27 °C.

To generate a Flag-tagged or GFP-tagged version of Kif24, human Kif24 cDNA was sub-cloned into pLVX-3xFlag-IRES-purovycin or pLVX-GFP-IRES-purovycin vector, respectively. To generate Kif24 mutants, Kif24 fragments encoding amino-acid residues 924–1,368 and 1–923, or full-length Kif24 with internal deletion mutants of amino-acid residues 951–631, 631–638 and 638–622 were amplified by PCR and sub-cloned into pLVX-3xFlag-IRES-purovycin or pLVX-GFP-IRES-purovycin vector. Human Nek2a cDNA was obtained from Dr Kunsoo Rhee (Seoul National University, South Korea) and sub-cloned into pLVX-Flag-IRES-purovycin or pLVX-GFP-IRES-purovycin vector, generating the respective flag-Kef24 or GFP-Nek2, respectively. To generate recombinant GST-Kif24, fragments of Kif24 encoding amino acids 1–218, 219–547, 548–678, 679–825, 831–941, 945–1,078, 1,087–1,129, 1,123–1,138, 1,148–1,187, 548–587, 588–594, 585–645, 639–657, 658–678, 679–717, 718–733, 734–788 and 789–825 were amplified by PCR and sub-cloned into pGEX-6p-1 vector encoding glutathione S-transferase (GST), and recombinant GST-Kif24, a Kif24 fragment spanning residues 93–1,368 was amplified and sub-cloned into pFastBAC vector. To generate FRET reporters, the mCerulean and mCitrine-containing MCA3T-ANT and MCAK-AC7 were amplified by PCR and sub-cloned into pEGFP-C1 vector, substituting the EGFP fragment. mCerulean alone, mCitrine alone, mCerulean-mCitrine, Kif24 and A220 (TS-a220) were also sub-cloned identically to the modified pEGFP-C1 vector. Pfu Turbo (Stratagene) and Phusion HF (NEB) polymerases were used for all PCR reactions. All constructs were verified by DNA sequencing. Expression of WT and mutant Kif24 or Nek2 in RPE1 and U2OS cells was carried out through lentivirus infection. Transient transfections of plasmids into HEK293 cells were performed using polyethyleneimine according to the manufacturer’s instruction.

Immunofluorescence microscopy. Indirect immunofluorescence was performed by fixing the cells with ice-cold methanol for 5 min on ice or with 10% formalin solution (Sigma-Aldrich) for 15 min at room temperature, and permeabilization with 0.5% or 1% Triton X-100, diluted in PBS, for 5 min at room temperature. Slides were blocked with the same serum albumin used for immunolabeling and incubated with primary antibodies. Secondary antibodies used were Cy3- Jackson Immunobulas) or AlexaFlour488- (Invitrogen) conjugated donkey anti-mouse or anti-rabbit IgG. Cells were then stained with 4,6-diamidino-2-phenylindole (DAPI), and slides were mounted using Prolong Gold anti-fade reagent (Life Technologies). Slides were observed and photographed using a Nikon Eclipse E800 microscope (Nikon; ×63 or ×100, numerical aperture (NA) 1.4) equipped with a Photometrics CoolSNAP HQ2 CCD camera. Images were acquired and processed with MetaMorph? (Molecular Devices). Percentage of cells with distal Nek2 puncta, cilia, elongated centroles and Ki-67 staining was quantified by first counting the total number of cells using 4,6-diamidino-2-phenylindole from a randomly selected field, and within the same field, switching filters to visualize and count the cilia expressing the marker of interest. Minimally, ten random fields were selected to be counted for a percentile.

Antibodies. Antibodies used in this study include polyclonal rabbit anti-Kif24 (ref. 21) (1:500 for western blot (WB) and 1:200 for immunofluorescence (IF)), anti-Nek2 (1:1,000 for WB and IF, cat. #610593, BD Biosciences), anti-Cep164 (1:1,000 for IF, cat. #ab2307, Abcam), anti-Nek2 (1:100 for WB and IF, cat. #610593, BD Biosciences), anti-Cep164 (1:1,000 for IF, cat. #04-1624, Millipore), anti-IFT88 (1:500 1:100 for WB, cat. #sc-8334, Santa Cruz Biotechnology), anti-glutamylated tubulin from Sigma-Aldrich, anti-GFP (1:1,000 for WB, cat. #G1544, Sigma-Aldrich and anti-centrin-2 (1:1,000 for IF, cat. #04-1624, Millipore), anti-Arl13b (1:100 for IF, from T. Katada and K. Kontani), and anti-Ki67 (1:1,000 for IF, cat. #ab15580, Abcam).

RNAi. Synthetic siRNA oligonucleotides were obtained from Dharmacon (GE Healthcare). Transfection of siRNAs using Lipofectamine RNAiMAX (Invitrogen) was performed according to the manufacturer’s instruction. For co-transfection of siRNA and FRET-Kif24, HEK293 cells were transfected with 2 μM siRNA and 2 μM of FRET-Kif24. FRET measurements were made on cells from three independent transfections, from each of which more than 100 cells were analyzed. Images were acquired using SoftWoRx suite software, and the mean fluorescence intensity was measured from each ROI using ImageJ to calculate the FRET ratio. In some cases, images were acquired using a DeltaVision Live Filter set (CFP and YFP) to obtain separate mCerulean and mCitrine images using a widefield fluorescence inverted microscope (Olympus IX-71) equipped with a Photometrics CoolSNAP HQ2 CCD camera. Images were acquired and processed with MetaMorph? (Molecular Devices). For co-transfection of siRNA and FRET-Kif24, HEK293 cells were transfected with 2 μM siRNA and 2 μM of FRET-Kif24. FRET measurements were made on cells from three independent transfections, from each of which more than 100 cells were analyzed. Images were acquired using SoftWoRx suite software, and the mean fluorescence intensity was measured from each ROI using ImageJ to calculate the FRET ratio. In some cases, images were acquired using a DeltaVision Live Filter set (CFP and YFP) to obtain separate mCerulean and mCitrine images using a widefield fluorescence inverted microscope (Olympus IX-71) equipped with a Photometrics CoolSNAP HQ2 CCD camera. Images were acquired and processed with MetaMorph? (Molecular Devices). For co-transfection of siRNA and FRET-Kif24, HEK293 cells were transfected with 2 μM siRNA and 2 μM of FRET-Kif24. FRET measurements were made on cells from three independent transfections, from each of which more than 100 cells were analyzed. Images were acquired using SoftWoRx suite software, and the mean fluorescence intensity was measured from each ROI using ImageJ to calculate the FRET ratio. In some cases, images were acquired using a DeltaVision Live Filter set (CFP and YFP) to obtain separate mCerulean and mCitrine images using a widefield fluorescence inverted microscope (Olympus IX-71) equipped with a Photometrics CoolSNAP HQ2 CCD camera. Images were acquired and processed with MetaMorph? (Molecular Devices).
measurements. Cells in which the probes were expressed in large aggregates or uniformly at high levels were excluded from the analysis.

FRET signals were calculated and normalized to donor emissions using the following formula: $I_{\text{f}}/I_{\text{d}}$ = (FRET – 2DONOR – 2ACCEPTOR)/DONOR where all fluorescence signals were corrected for background fluorescence. $x$ is the fraction of mCerulean donor signal detected in the FRET channel and $\beta$ is the fraction of a mCitron acceptor signal seen in the FRET channel after excitation with 490 nm light. For the microscope used in our studies, we determined that $x = 0.96$ and $\beta = 0.15$.

**Statistical analysis.** The statistical significance of the difference between two means was determined using a two-tailed Student's t-test. Differences were considered significant when $P < 0.05$.

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**Acknowledgements**

We thank members of the Dynlacht laboratory for engaging discussions and encouragement and H. Zheng (NIA) for mass spectrometric analysis. We are indebted to Dr Y. Deng and Mr M. Cammer for their help with FRET analysis, Dr C. Walczak for FRET reporter constructs, Dr K. Rhee for Nek2 plasmids, and Drs E. Lee, T. Tang, T. Kataka and K. Kontani for antibodies used throughout this study.
This work was supported by NIH grant R01HD069647 and was initiated with support from a March of Dimes grant to B.D.D.

Author contributions
S.K. and B.D.D. designed the research. S.K., K.L., J.-H.C. and B.D.D. performed the experiments. S.K. and B.D.D analysed the data. S.K., J.-H.C. and N.L. designed the FRET-related experiments. S.K. and B.D.D. wrote the paper and supervised the project. All authors participated in the discussion of the data and production of the final version of the manuscript.

Additional information
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How to cite this article: Kim, S. et al. Nek2 activation of Kif24 ensures cilium disassembly during the cell cycle. Nat. Commun. 6:8087 doi: 10.1038/ncomms9087 (2015).

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