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Titolo:

# A novel Rab5-based signalling pathway participates in centrosome cohesion.

Tesi presentata da:
***Valentina Margaria***

Tutor:
***Prof. Federico Bussolino***

Coordinatore del Dottorato:
***Prof. Federico Bussolino***

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**I am very grateful to my family, my friends and *Riccardo* for all the help they have given me.**

# Abstract

The centrosome is a non-membranous organelle that acts primarily as a microtubule organising centre. During interphase, centrosomes organise the microtubule network responsible for vesicular transport, for cell shape and polarity. At mitosis, centrosomes direct the formation of the mitotic spindle and ensure proper separation of replicated chromosomes.

The centrosome is composed of two paired orthogonal centrioles that replicate at the G1/S transition giving rise to two centrosomes that are held together until G2 when they separate migrating to the opposite poles of the cell.

The control of centrosome cohesion is an important aspect of cell division, since its deregulation can affect spindle assembly. Not surprisingly, therefore, deregulation of centrosome division and dynamics is thought to play a major role in genomic instability associated with tumorigenesis.

Here we report the identification of a novel Rab5-dependent pathway participating in centrosomes separation.

Rab5 is a small GTPase involved in the control of intracellular trafficking, homeostasis of the endosomal compartment and actin cytoskeleton remodelling. The activity of Rab5 is tightly regulated by GDP/GTP exchange factors (GEFs), like Rabex-5, and GTPase activating protein (GAPs), such as RN-tre.

We found that Rab5, RN-tre and Rabex-5 localise at the centrosome in human cells. Moreover increased Rab5 activity caused loss of centrosome cohesion suggesting a role for this GTPase in centrosome function. Indeed, reduction of the Rab5 activity inhibited centrosome separation during G2 and decreased the distance between the spindle poles at mitosis.

The molecular mechanism appears to involve the kinesin motor protein KIF3A. KIF3A binds to RN-tre and its depletion prevented the loss of centrosome cohesion caused by excess of active-Rab5.

More importantly, KIF3A silencing phenocopies the effects of Rab5 depletion, inhibiting centrosome separation and causing defective spindles.

Thus KIF3A has the characteristics of a Rab5-downstream effector and participates in the separation of duplicated centrosomes.

# Introduction

## *The centrosome*

The centrosome has fascinated cell biologists for over a century since the pioneering studies of Boveri put this organelle in the spotlight (Boveri, 1914).

The main function of the centrosome is to organize a dynamic array of microtubules (MTs) (Bornens, 2002; Job et al., 2003) MTs are polymers composed of α- and β-tubulin subunits and their growth is initiated by γ-tubulin ring complexes (γTuRCs), which nucleate microtubules.

During interphase of the cell cycle, MTs determine cell shape, polarity and motility, whereas, during M phase, they form the bipolar spindle required for chromosome segregation. The single centrosome present in a G1-phase cell comprises two centrioles embedded in a protein matrix known as pericentriolar material (PCM). Before division into two daughter cells, this whole structure needs to be duplicated once, so that a G2-phase cell harbours two centrosomes each comprising two closely linked centrioles. **(Figure 1)**

The centrioles and PCM define the centrosome as one of the most complex non membranous organelles in the cell.

Centrioles are tiny, barrel-shaped structures that are related to basal bodies, which, in turn are essential for the formation of cilia and flagella. In vertebrates the centrioles are composed of nine triplets microtubules, whereas in Drosophila and Caenorhabditis elegans they mostly comprise doublet and singlet microtubules, respectively (Delattre and Gonczy, 2004). **(Figure 2)**

The PCM surrounding the centrioles has been visualised as fibrous lattice (Dictenberg et al., 1998) and, in a human centrosome, contains over 100 different proteins (Andersen et al., 2003). These include components required for microtubule nucleation, notably γ-tubulin, and associated proteins that are conserved in evolution (Winey and O'Toole, 2001). Other PCM components are less conserved, although many harbour predicted

coiled-coil domains (Andersen et al., 2003), suggesting that they perform scaffolding functions for the recruitment of cell cycle regulatory proteins (Doxsey et al., 2005; Sluder, 2005)

The ultimate purpose of mitosis is the generation of genetically identical daughter cells. All chromosomes are replicated during S phase, and the resulting pairs of sister chromatids must be segregated equally during mitosis. The fidelity of this process depends on the assembly of a strictly bipolar mitotic spindle. This essential event is dependent upon the reproduction or duplication of the centrosome (Compton, 2000; Mazia, 1987).

Therefore, the cell cycle regulatory machinery must control not only a chromosome cycle but also a centrosome-centriole cycle; the integration between the two cycles is crucial for genome stability (Mazia, 1987). Any deviation from normal centrosome numbers can results in the formation of mono- or multi-polar spindles, with dire consequences for the accuracy of chromosomes segregation. Accordingly, centrosome abnormalities have long been related to aneuploidy and proposed to contribute to the development of cancer (Brinkley, 2001; Doxsey, 1998; Sluder and Hinchcliffe, 1999; Urbani and Stearns, 1999; Zimmerman et al., 1999).

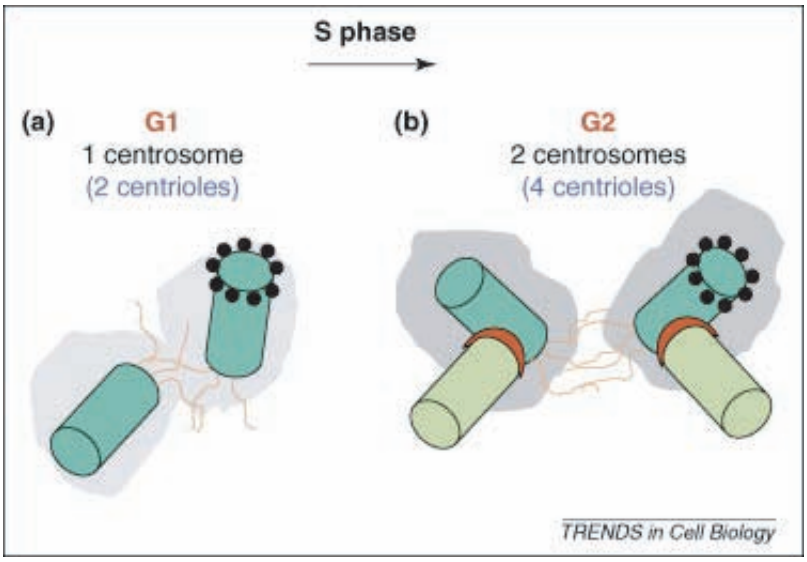
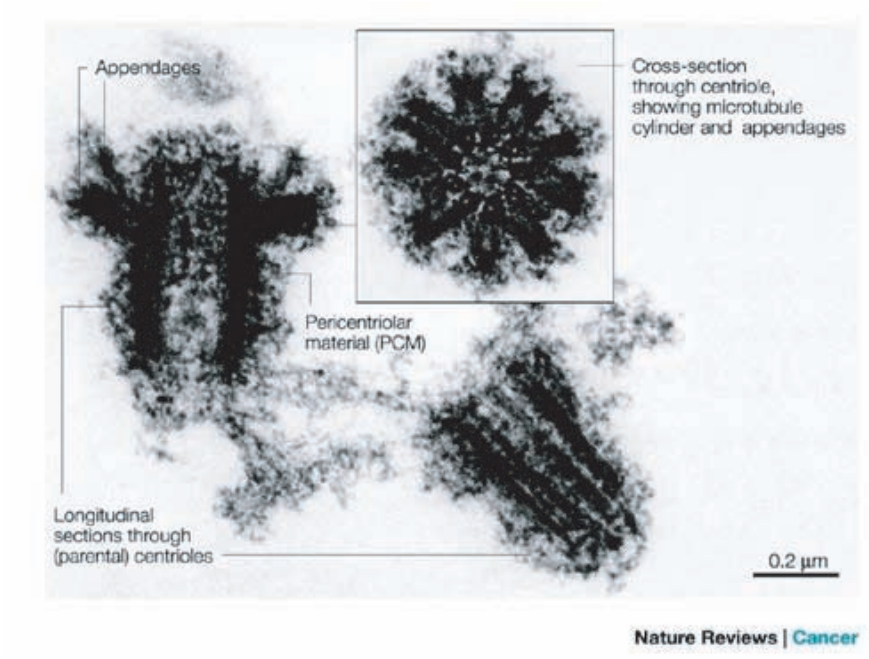



**Figure 1.** ***Centrosomes in G1 and G2 phase cells.***

(a) A cell in G1 phase of the cell cycle harbours a single centrosome comprising two centrioles (green). The two centrioles are structurally distinct, reflecting their different ages: the older one (a 'parent' in the preceding cell cycle) carries distal and subdistal appendages (indicated in black), structures implicated in both MT anchoring and ciliogenesis, whereas the younger one (a 'progeny' formed during the preceding cell cycle) lacks appendages. Both centrioles are embedded in PCM (grey). During G1, the two centrioles are tethered to each other only loosely, apparently through entangling fibers associated with their proximal ends ('baseto- base' association; brown lines).
(b) In the following S phase both centrioles then give rise to progeny, so that by G2 phase the cell harbours two centrosomes, each made up of two tightly associated centrioles. Within each centrosome, parent and progeny centriole display a close orthogonal association ('base-to-side'; red disks); according to a recent model, this association (termed 'engagement') prevents reduplication in the same cell cycle. Note that the PCM undergoes a phosphorylation-dependent 'maturation' event in late G2 (indicated by dark grey). This enhances the recruitment of γ-tubulin ring complexes and allows the increased MT nucleation activity required for spindle formation (Nigg, 2007)

**A**

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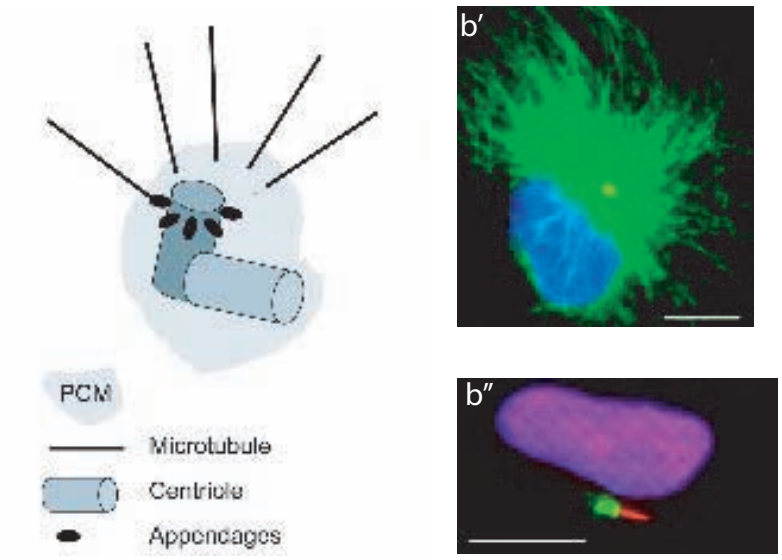



**Figure 2.** ***The vertebrate centrosome.***

A) Electron microscopy view of the centrosome: longitudinal section and cross section. (Nigg, 2002)
B) Schematic view of 1 vertebrate centrosome. Shown are 2 centrioles (barrels of triplet MTs) embedded within the pericentriolar material (PCM), from which MTs are nucleated. The structure shown here emphasizes the near orthogonal orientation of the paired centrioles that is characteristic of the centrosome from the time of duplication until early mitosis.
(b')A single centrosome in a cultured HeLa cell, as visualized by immunofluorescence microscopy using antibodies against g-tubulin (yellow); MTs are stained in green.
(b'') Double-staining of human lung adenocarcinoma cell (A549) with antibodies against pericentrin and polyglutamylated tubulin. This staining allows visualization of the growth of a primary cilium in response to arrest in a quiescent state (Go). Note that the primary cilium grows off one of the 2 centrioles. Using appropriate markers, this centriole can be identified as the mature centriole. (Nigg, 2006)

Figure 2

## The centrosome cycle and its regulation

The centrosome-centriole duplication cycle is traditionally subdivided into discrete steps: *centriole disengagement, centriole duplication, centrosome maturation* and *centrosome separation* [reviewed in (Nigg, 2007)]. **(Figure 3)**

In a metaphase cell, each of the two spindle poles is characterised by the presence of one centrosome comprising two centrioles. These two centrioles represent a parent-progeny originating from the previous cell cycle: they are tightly associated with each other and usually have an orthogonal arrangement.

The tight link between the two centrioles is lost during early G1 phase, in a process referred to as “disengagement” (Tsou and Stearns, 2006b; Vidwans et al., 1999). Importantly, this disengagement is proposed to license the two centrioles for a new round of duplication (Tsou and Stearns, 2006a).

During G1, a different, highly dynamic linker structure is established between the two centrioles. Centriole duplication begin during S phase; one new centriole, procentriole, starts to grow at an orthogonal angle next to each licensed centriole, again establishing tight connections between parental and progeny centrioles. The two procentrioles then elongate until they reach full length in G2 and, in late G2, the younger of the two parental centrioles acquires appendages, thereby reaching full maturity. At about the same time, the loose tether between the two parental centrioles is severed and enables centrosome separation and spindle formation.

Several vertebrates’ kinases have been implicated in centrosome duplication (Sluder and Nordberg, 2004).

The most definitive evidence supports a role for Cdk2-Cyclin A and/or – Cyclin E (Matsumoto et al., 1999; Meraldi and Nigg, 2001; Tsou and Stearns, 2006b). However, a detailed mechanistic understanding of the Cdk requirement for centrosome duplication has not yet emerged. Thus, until a direct action of Cdk2-Cyclin A or E at the centrosome can be demonstrated, it remains possible that Cdk activity is required primarily to advance cells to a cell cycle state that permits centrosome duplication.

While this issue remain unresolved, a member of the Polo kinase family, Plk4, has unequivocally been identified as a positive regulator of centriole duplication in both human cells and Drosophila (Habedanck et al., 2005). In the absence of Plk4 activity, both vertebrate and invertebrate cells progressively lose centrioles through impaired duplication, leading to severe anomalies in spindle formation (Burke et al., 2001). Plk4 has previously been shown to be essential for embryogenesis in mice (Hudson et al., 2001) and, interestingly, Plk4+/- mice are prone to develop tumours (Ko et al., 2005). It is of particular interest that the overexpression of Plk4 in human cells results in the production of multiple centriole precursors surrounding a single parental centriole (Habedanck et al., 2005).

The question of how cells keep centriole numbers constant over successive cell division continues to represent one of the most mysterious problems in contemporary cell biology.

When considering the centrosome cell cycle from a purely conceptual perspective, one can discern two distinct rules: i) *the cell cycle control* that stipulates that centrosome duplicates once and only once in every cell cycle, ii) *the copy number control* that governs the formation of only one progeny centriole next to each parental centriole. Although conceptually distinct, these two modes of control are expected to be coordinated at the molecular level in order to avoid to give rise to aberrant centriole numbers and, consequently, to genome instability. **(Figure 4)**

An intriguing model for *the cell cycle control* has been proposed by Tsou and Stearns; their data suggest that Separase, a protease already well-known for its role in chromatid separation (Uhlmann et al., 2000), is also required for centriole disengagement and that this event is in turn crucial for the subsequent growth of new centrioles.

In this scenario the centriole engagement, established during centriole duplication in S phase, prevents further duplication in the same cell cycle until passage through M phase (Tsou and Stearns, 2006b).

At present a more detailed definition of the pathway that leads to centrioles disengagement is not available.

*The copy number control* seems to have Plk4 as a major regulator and it has been suggested that procentriole formation might critically depend on

the phosphorylation of one or more proteins at the procentriole assembly site. To better understand this model a lot of studies are concentrated on the characterisation of the direct substrate(s) for Plk4 and on the identification of the phosphatase(s) that is expected to counteract this kinase.

At the end of the centrosome cycle, centrosome separations together with centrosomes segregation lead to the formation of the mitotic bipolar spindle. How centrosome cohesion is regulated during the cell cycle is not well understood, but both cytoskeletal dynamics (Euteneuer and Schliwa, 1985; Thompson et al., 2004) and regulatory protein phosphorylation have been implicated. It has been suggested that centrioles are connected by linker structures (Bornens et al., 1987; Paintrand et al., 1992) , but the existence of in vivo linkers remains hypothetical, and their composition is unknown.

The centrosomal coiled-coil protein C-Nap1 has been proposed to provide a docking site for a putative linker (Fry et al., 1998a; Fry et al., 1998b; Mayor et al., 2000). At the onset of mitosis, the inhibition of the protein phosphatase 1$\alpha$ (PP1$\alpha$) (Helps et al., 2000; Meraldi and Nigg, 2001) is thought to enhance the phosphorylation of C-Nap1 by the protein kinase Nek2 (Faragher and Fry, 2003; Fry et al., 1998a; Fry et al., 1998b), causing its functional inactivation and, ultimately, its dissociation from the centrosome (Mayor et al., 2002; Mayor et al., 2000). Recently, another protein distantly related to C-Nap1, Rootletin, has been shown to be involved in the maintenance of centrosome cohesion (Bahe et al., 2005).

Nek2 is not the only kinase implicated in the regulation of centrosome cohesion. Meraldi and Nigg demonstrated that also Cdk2, in association with either cyclin A or E is able to induce centrosome splitting (the separation of parental centrioles) possibly in preparation for centrosome duplication (Meraldi and Nigg, 2001). In particular they proposed a model in which the balance of centrosome-associated kinase and phosphatase activities constitutes a major mechanism to determine centrosome dynamics during cell cycle.

At the onset of mitosis the assembly and the maintenance of the bipolar spindle depend on force-generating motor proteins.

Eg5 is a motor protein belonging to an evolutionarily conserved family of plus-end directed, bipolar kinesins, whose founding member is the product of the bimC gene in *Aspergillus nidulans* (Enos and Morris, 1990). The central role of Eg5 in centrosome separation has been well established: Eg5 phosphorylation, exerted by Cdk1, allows the interaction between the motor protein and the duplicated centrosomes resulting in their separation at the onset of mitosis (Blangy et al., 1995).

Cells treated with monastrol, a small molecule targeting specifically the mitotic kinesin Eg5, arrested in mitosis with monoastral spindles, comprised of a radial array of microtubules surrounded by a ring of chromosomes (Kapoor et al., 2000).

Several studies have underlined the involvement of regulatory proteins in the formation of bipolar spindle. Recently, Di Fiore et al. demonstrated that the overexpression of RanBP1, a major effector of Ran GTPase, induces multipolar spindles. Specifically, RanBP1 excess perturbs cohesion of centrioles within diplosomes in mitosis generating isolated centrioles that can individually organize functional spindle poles. The splitting activity of RanBP1 requires microtubule integrity and Eg5 activity (Di Fiore et al., 2003).

Further investigations are needed to discover other crucial factors that control structural and dynamic features of centrosomes during mitosis.

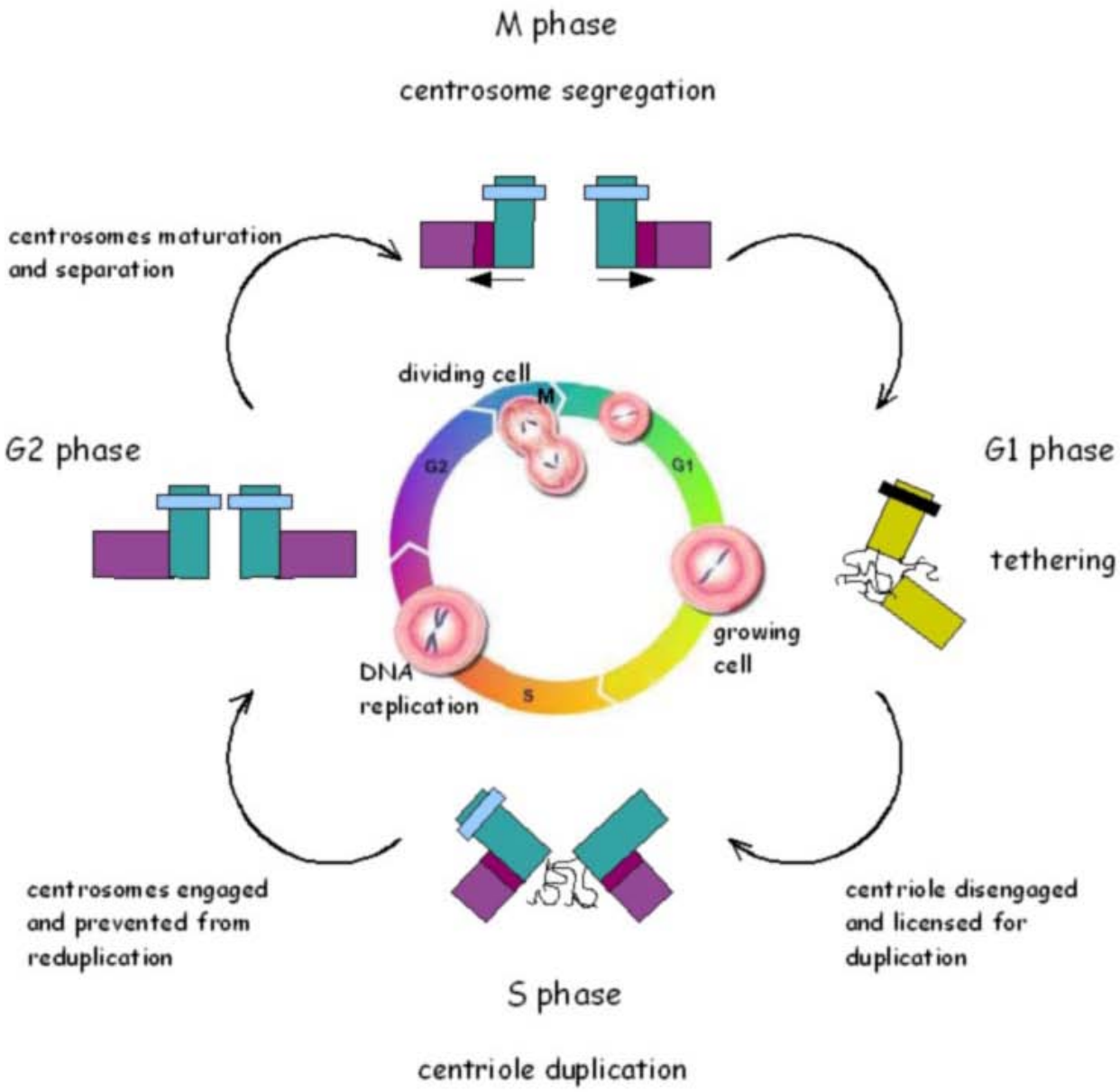



**Figure 3.** ***Schematic view of centrosome and cell cycle.***

This scheme summarizes the key steps in the centrosome cycle during the indicated stages of the cell cycle (M, G1, S and G2). During the M phase, 1 centrosome (comprising 2 centrioles) is present at each spindle pole. Upon exit from mitosis, the 2 centrioles within each mitotic centrosome undergo disengagement (i.e., they loose their characteristic orthogonal association), and recent data indicate that disengagement constitutes a necessary _licensing_ step for centriole duplication during the subsequent S phase. In addition, disengagement is thought to allow the establishment of a dynamic tethering structure connecting the 2 (future parent) centrioles to each other. Note that the 2 centrioles present in a G1 phase cell are not equal. Only 1 (the older one) is fully mature, as indicated by the presence of appendages at its distal end (black bar). During S phase, exactly 1 procentriole forms at an orthogonal angle at the proximal end of each parental centriole. These procentrioles then grow until 2 pairs of centrioles are present in G2 phase. Throughout these cell cycle stages, the close association of the newly generated centrioles with their respective parents (engagement) is thought to prevent a second round of duplication. Moreover, tethering between the 2 parental centrioles ensures that the duplicated centrosomes continue to function as a single MTOC. Late in G2, this connecting tether is then severed and centrosomes undergo maturation (red bars). Subsequently, the separated centrosomes move apart to associate with the poles of the forming mitotic spindle. (Nigg, 2002)

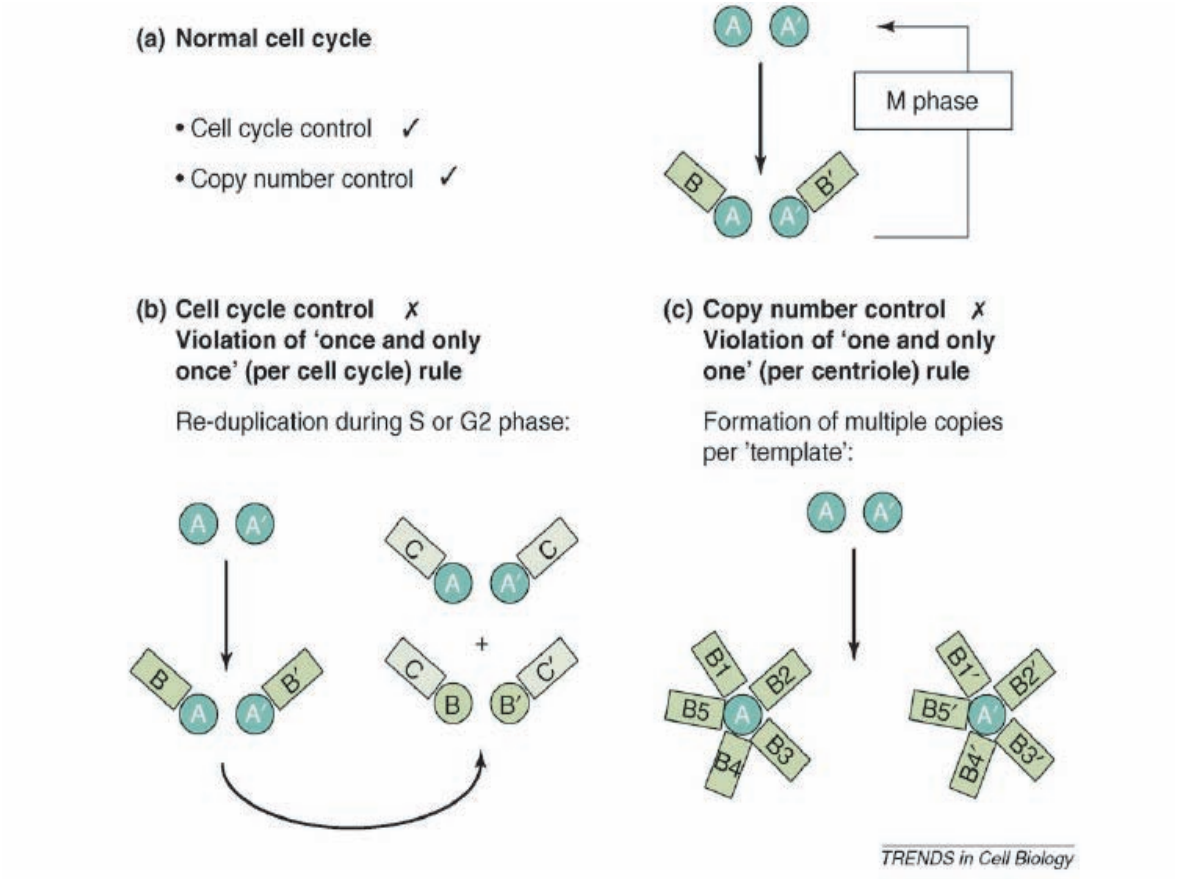



**Figure 4.** ***Two rules governing the centrosome cycle.***

(a) Centriole duplication in a normal cell cycle involves two centrioles (A and A0) giving rise to progeny (B and B0). This process is proposed to be controlled by two mechanisms.
(b) The first mechanism imposes cell cycle control and ensures that a new round of duplication can occur only after passage through M phase. Violation of this 'once and only once' per cell cycle rule results in reduplication during S or G2 phase, leading to extra centrioles (C and C0).
(c) The second mechanism imposes copy number control at each duplication event and limits the formation of procentrioles to one per pre-existing centriole. Violation of this 'one and only one' per centriole rule results in the formation of multiple (pro)centrioles (B1–B5 and B10–B50) per template. Deregulation of either cell cycle control or copy number control has the potential to produce excessive numbers of centrioles, a phenotype commonly observed in cancer cells

## Interrelationship between centrosome cycle and cell cycle

A growing body of evidence indicates that centrosomes function as multiplatform scaffolds for a multitude of signalling networks (Doxsey et al., 2005).

As many regulators are found at centrosomes, it is tempting to speculate that centrosomes serve as solid-state signalling machine capable of regulating many cellular functions, although, in most cases, the function of the centrosome-anchored fraction of these molecules has not been determined. Accurate cell division requires the coordination of two separate but interdependent cycles: the cell cycle, and the centrosome cycle. **(Figure 3)**

Most results demonstrate a requirement for centrosomal anchoring of regulatory pathways in the control of cell-cycle progression. Different studies have provided evidence to link centrosome activity to the completion of cell division (cytokinesis) and to G1-S transition.

The central role of the mitotic spindle in defining the site of cell cleavage during cytokinesis has long been known (Rappaport, 2006). In separate studies Hinchcliffe et al. and Khodjakov and Ryder demonstrated that acentrosomal cells failed to complete cell division, these cells remained attached by thin intercellular bridges or aborted cytokinesis to form single binucleate cells (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). Using time lapse microscopy to better understand this cleavage furrow failure, Piel et al. found out that the movement of the maternal centriole to the intercellular bridge may be required for completion of cytokinesis (Piel et al., 2001). Thus, the centrosome could directly activate cell division. Another open possibility is that the centrosome could release cells from arrest at a cytokinesis checkpoint (Doxsey, 2001).

Furthermore, acentrosomal cells, followed after mitosis, seemed to arrest in G1 phase and did not initiate DNA replication. One interpretation of this block in DNA replication is that centrosomes directly activate or concentrate factors that are essential for the initiation of DNA synthesis. Consistent with

this idea is the centrosomal localisation of molecules that control entry in S phase (e.g. Cyclin E) (Hinchcliffe et al., 1999; Lacey et al., 1999).

An alternative explanation is that animal cells monitor the presence of centrosomes, and, in their absence, activate a checkpoint that prevents the onset of S phase.

Although the pathway that activates G1 arrest in acentrosomal cells has not yet been characterised, the emerging picture is that the centrosome can exert control over the cell cycle. This suggests that the interrelationship between centrosome and cell cycle might be required for transitions between several cell cycle stages.

The most enticing idea is that centrosomes might anchor signal-transduction pathways and serve as a central site that receives and integrates signals from outside the cell and facilitates the conversion of these signals into cellular functions in the cell interior (Doxsey et al., 2005).

## Centrosome aberration and the development of cancer

Recent studies have implicated centrosome amplification in the origin of chromosomal instability during tumour development. Theodor Boveri first suggested this notion nearly a century ago (Boveri, 1914).

Deregulation of the centrosome cycle, or loss of coordination between the centrosome cycle and the chromosome cycle, will almost inevitably lead to a change in ploidy (the number of homologous sets of chromosomes) or chromosomal instability (D'Assoro et al., 2002a; D'Assoro et al., 2002b; Nigg, 2001; Nigg, 2006).

Centrosome abnormalities in cancer cells have been extended to a large number of different human tumours (Brinkley, 2001; Duensing, 2005; Lingle et al., 2002; Nigg, 2002; Pihan et al., 2003).

Centrosome abnormalities may be classified as either “structural” or “numerical” and, although the two types often occur together, their origins as well their consequences may differ.

Structural centrosome aberrations, most likely, arise through deregulated expression of genes coding for centrosomal components or altered

posttranslational modifications (e.g. phosphorylation). Centrosomes then appear altered in size, or show abnormal orientation or location. Moreover, one can often observe the formation of centrosome related bodies (CRBs) (Casenghi et al., 2003; Fry et al., 1998b). These structural centrosomal abnormalities altered centrosome function in regulating shape, polarity and motility of the cell (Jiang et al., 2003). Thus, they have been implicated as a potential cause of loss of cell and tissue architecture seen in cancer (e.g. anaplasia) (Jiang et al., 2003).

Numerical centrosome aberrations often correlate with genome instability and loss of tissue differentiation (Lingle et al., 2002; Ghadimi et al., 2000; Kronenwett et al., 2005), and have also been proposed to have prognostic value in tumour progression (Yamamoto et al., 2004). **(Figure 6)**

Supernumerary centrosomes can arise through a number of different mechanisms, such as over duplication, cytokinesis failure, cell fusion or *de novo* genesis. **(Figure 5)**

It has been demonstrated that centrosome amplification in cancer cells can develop through dysfunction of alternative pathways that converge on G1/S and G2/M checkpoint regulators (D'Assoro et al., 2002).

G1/S and G2/M checkpoints are surveillance mechanisms that enforce dependency on the orderly completion of cell cycle events (Hartwell and Weinert, 1989). When activated, these checkpoints inhibit the formation and/or activation of Cdks and thereby induce cell cycle arrest (Murray, 1992).

Several key proteins involved in checkpoint control also physically associate with centrosomes and appear to play an important role in centrosome homeostasis (Brown et al., 1994; Giannakakou et al., 2000; Hsu and White, 1998; Morris et al., 2000; Pockwinse et al., 1997; Xu et al., 1999).

The tumour suppressor p53 controls both G1/S and G2/M checkpoints and its inactivation also lead to disregulation of the centrosome cycle (Tarapore and Fukasawa, 2002).

In human cancers, p53 mutations correlated with the occurrence of centrosome amplification in carcinomas of the breast, head, neck and

prostate, and in neuroectodermal tumours (Carroll et al., 1999; Ouyang et al., 2001; Weber, 1998).

In some cases tumours that retained wild-type p53 also showed amplified centrosomes. Many of these tumours overexpressed Mdm2 that inactivates p53 by promoting its degradation (Carroll et al., 1999).

Centrosome amplification and genomic instability can develop independently of loss of p53 function, suggesting the presence of alternative mechanisms leading to disregulation of centrosome homeostasis (Donehower et al., 1992; Eshleman et al., 1998; Lengauer et al., 1997; Lingle et al., 2002).

The high risk human papillomavirus (HPV) types 16 and 18, which each carry two viral oncogenes, E6 and E7, have been implicated in the induction of centrosome amplification in human cell lines (Duensing, 2001; Duensing et al., 2000). HPV E6 and E7 may interfere with centrosome homeostasis by targeting different pathways. Whereas E6 may operate through inactivation of p53 function, E7 lead to centrosome amplification through inactivation of the Rb and G1/S checkpoint disregulation (Duensing and Munger, 2002).

Moreover, mutations in the BRCA1 and BRCA2 tumour suppressor genes, associated with the development of familial breast and ovarian cancers, have also been implicated in the loss of checkpoint control of the centrosome cycle (Deng, 2002). Mouse embryo fibroblast, carrying gene targeted deletions in BRCA1 and BRCA2, showed defective G2/M checkpoint function, amplified centrosomes, aberrant mitosis and aneuploidy (Tutt et al., 1999; Xu et al., 1999). Taken together these studies emphasise the role of tumour suppressor genes as centrosome regulators.

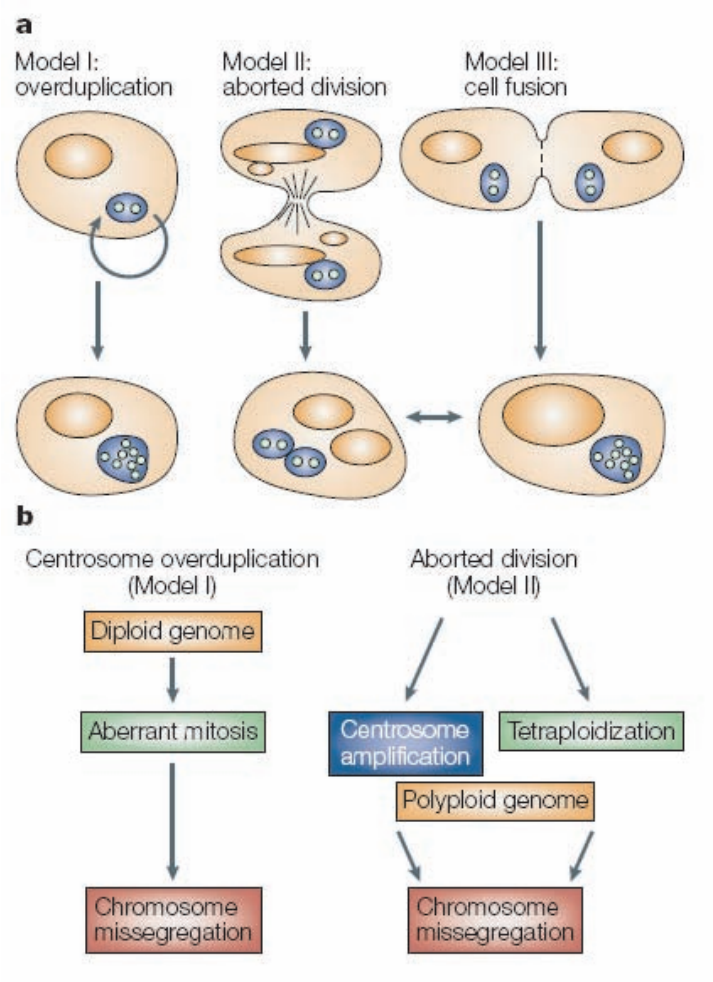



**Figure 5.** ***Centrosome amplification.***

(a) Mechanisms of centrosome amplification. Three plausible models for the generation of supernumerary centrosomes. A fourth model — de novo assembly of centrioles — is not indicated. For the sake of simplicity, all supernumerary centrosomes are shown in clusters, although scattered distributions might also be generated. Model I: deregulated centrosome duplication. Supernumerary centrosomes arise through several rounds of duplication within a single S phase. Model II: failure to complete cell division. As a result of an aborted mitosis, a tetraploid (or near-tetraploid) cell contains two centrosomes that are already in G1. Model III: cell fusion. Depending on the cell-cycle stages of the fusion partners, the products of such fusions will display different centrosome/ genome ratios. Note that the products of fusion and aborted cell division will first be multinucleated, but often form single polyploid nuclei after subsequent mitoses.
(b) Centrosome amplification and ploidy. Centrosome overduplication during a prolonged S phase will give rise to supernumerary centrosomes in a diploid cell. In striking contrast, an aborted mitosis will generate supernumerary
centrosomes that are concomitant with an increase in ploidy. Although supernumerary centrosomes are expected to cause chromosome missegregation in all dividing cells (regardless of ploidy), the likelihood of generating viable, potentially harmful progeny (in the form of hyperdiploid cells) is enhanced when segregating chromosomes of a tetraploid rather than a diploid genome. So, the combination of supernumerary centrosomes with tetraploidy sets the stage for chromosome missegration and chromosomal instability. (Nigg, 2002)

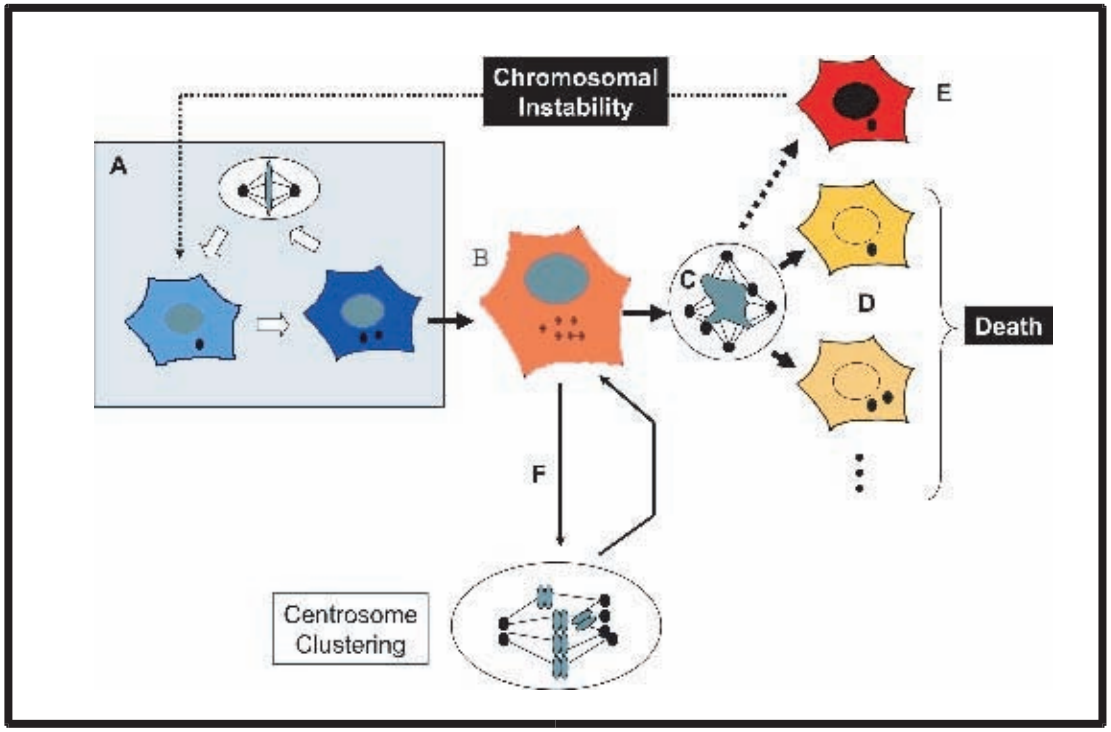



**Figure 6.** ***Predicted fates of cells harboring supernumerary centrosomes.***

Disturbance (by any one of the mechanisms described in Fig. 5) of the normal centrosome duplication-segregation cycle (depicted in (A)) will give rise to cells harboring multiple centrosomes (B). These will frequently
undergo multipolar divisions (C), resulting in extensive chromosome-missegregation and, most commonly, progeny that harbors nonviable
chromosome constellations (D). Occasionally, though, cells will arise that have not only a normal centrosome number restored, but also, by chance, an aberrant (aneuploid) complement of chromosomes that confers a selective advantage (E). Such cells will be able to re-enter the proliferating pool. Assuming that this pool of cells still carries the lesion(s) that gave rise to the original centrosome abnormality, the process of genome destabilization (chromosomal instability) will continue. As illustrated in (F), multipolar divisions are not an inevitable consequence of supernumerary centrosomes. Most cells are in fact endowed with a centrosome-independent mechanism for bipolar spindle formation. Perhaps as a consequence of upregulation of this alternative pathway for bipolar spindle formation, some tumor cells may undergo frequent bipolar divisions in spite of supernumerary centrosomes. In these cases, multiple centrosomes can be seen to cluster at the 2 (usually broad) poles of bipolar spindles. As suggested in (F), there is a suspicion that such spindles will segregate chromosomes with reduced fidelity. (Nigg, 2006)

## *Trafficking proteins and cell cycle progression*

A growing body of evidence is implicating endocytic/trafficking proteins in the regulation of cellular processes that would appear *prima facie* unrelated to intracellular membrane traffic.

Cells possess an elaborate and highly structured signal transduction machinery to translate responses to external stimuli into programmed changes in gene expression. In recent years it has become clear that the output of a signalling process depends not only on activation of a particular set of signalling molecules, but also on where and for how long the signal is emitted.

Exciting new findings suggest that the signalling machinery can achieve a high level of regulation by exploiting the compartmentalisation and functional specialisation of the endocytic pathway, in order to control processes such as cell fate determination, cell migration and cell cycle progression (Miaczynska et al., 2004).

One obvious role for endocytosis in signalling is to provide temporal regulation, as the duration of the signalling process depends on both the kinetics of receptors internalisation and on the proportion of receptors undergoing degradation compared to those recycling to the plasma membrane (Wiley and Burke, 2001; Wiley, 2003). Endosomes may provide another level of signalling control, a spatial regulation, selectively targeting molecules to specific organelles and segregating them into specialised subdomains, such as synapses in lymphocytes (Tseng and Dustin, 2002), and focal adhesion sites in epithelial cells (Bershadsky et al., 2003).

Different proteins, which have been well characterised for their role in endocytosis, are now emerging as regulators of mitotic progression, spindle assembly, cytokinesis, centrosomes cohesion and primary cilia formation.

One such case is represented by Rab6A', which regulates trafficking between the Golgi and post-Golgi compartments (Goud et al., 1990; Antony et al., 1992). Rab6A' has also been indicated in the regulation of metaphase progression, since it cooperates with the Mad2-dependent spindle checkpoint pathway, ensuring that the spindle microtubules attach to the kinetochores at metaphase (Miserey-Lenkei et al., 2006).

Another example is represented by Clathrin, a major component of coated pits, which has an established function in the generation of vesicles that transfer membrane and proteins around the cell (Brodcky et al., 2001; Kirchhausen et al., 2000; Robinson et al., 2004). During mitotic progression Clathrin stabilises fibres of the spindle to aid congression of chromosomes, thus its depletion prolongs mitosis and activates spindle checkpoints such as Mad2 (Royle et al., 2005).

Furthermore, Rab11 that acts as a regulator of vesicles transport through the recycling endosomes has been recently recognised as an essential player for completion of abscission in the terminal steps of cytokinesis. Moreover, the Evi5 oncogene, a putative Rab GTPase activating protein (GAP), has been identified as a binding protein for Rab11 (Westakle et al., 2006).

In previous studies Evi5 had been shown to be a novel centrosomal protein (Faitar et al., 2005), involved in the regulation of cell cycle progression (Eldridge et al., 2006) and in the final stages of cell division (Faitar et al., 2006). Thus, it is expected that Rab11 and Evi5 cooperate to coordinate vesicular trafficking, cytokinesis and cell cycle control.

We can also mention Dynamin2 (Dyn2), a large GTPase involved in vesicle formation and actin reorganisation (Hinshaw, 2000; McNiven et al., 2000; Orth and McNiven, 2003). Dyn2 localises to the spindle midzone where it plays an essential role in the final regulation of dividing cells (Thompson et al., 2002). Dyn2 also localises at the centrosome and participates in centrosome cohesion (Thompson et al., 2004).

Finally, Rab8a has been described as being involved in primary cilia formation through its specific binding with cenexin/ODF2, a basal body protein that is involved in the nucleation of microtubules at the centriole and is required for cilium formation (Yoshimura et al., 2007).

In other instances, however, endocytic/trafficking proteins seem to participate in events apparently unrelated to membrane dynamics (Benmerah et al., 2003; Vecchi and Di Fiore, 2005).

Prompted by this background, and by the finding that Rab5 and its regulators, RN-tre and Rabex5, are localised at the centrosome (see below), we investigated a possible role for this membrane traffic-controlling GTPase in the regulation of centrosome physiology.

## *Rab5, a master regulator of endocytosis*

The endosomal system represents an interconnected and dynamic network of organelles, which differ in their biochemical composition and localisation within the cell.

Endocytic organelle exhibits a complex morphological organisation in the form of membrane vacuoles, cisternae, tubules and multilamellar or multivesicular bodies.

Molecules internalised at the plasma membrane, by means of clathrin dependent or clathrin independent endocytosis, reached early endosomes. Proteins internalised in the early endosomes could either be sorted into late endosomes and lysosomes for degradation, or recycled to the plasma membrane passing through the pericentriolar recycling endosomes (Lemmon and Traub, 2000).

The complexity of these transport pathways implies that each endocytic compartment must possess specific molecular machineries that enable fusion of different incoming vesicles destined to various organelles. Furthermore, dynamic interaction of the endocytic compartments with the cytoskelston enables the motility of transport vesicles and the positioning of organelles within the cell and likely influence organelle shape (Kamal and Goldstein, 2000; Miaczynska and Zerial, 2002). **(Figure 7)**

Rab GTPases, members of the Ras superfamily of small GTPases, are key regulators of membrane trafficking and receptor localisation in eukaryotic cells (Zerial M. & McBride H., 2001; Pfeffer S. & Aivazian D., 2004).

Rab proteins constitute the largest family of monomeric small GTPases; eleven Rab (Yptp/Sec4p) proteins are expressed in Saccharomyces cerevisiae but there might be as many as 70 family members in human (Colicelli, 2004; Zerial and McBride, 2001). This increased complexity through the evolution reflects a greater need for cell organisation.

The Rab proteins are localised to the surfaces of distinct membrane-bound compartments and regulate transport vesicle formation and internalisation, motility, docking and fusion (Zerial M. & McBride H., 2001; Pfeffer S. & Aivazian D., 2004; Gonzales and Scheller, 1999; Mohrmann and van der Sluijs, 1999; Schimmoller et al., 1998).

The regulatory principle of Rab proteins, as for other GTPases, lies in their ability to function as molecular switches that oscillate between GTP (the active form) and GDP bound conformation. Nucleotide exchange is tightly regulated by specific Guanine Nucleotide Exchange Factors (GEFs) that activate the Rabs, and GTPase Activating Factors (GAPs) that, by stimulating GTP hydrolysis, induce Rab proteins inactivation.

In addiction to cycling between GTP-and GDP-bound states, Rabs cycle between the membrane and cytosol. This latter cycle depends on GDI (Rab guanine nucleotide dissociation inhibitor), which functions as a Rab vehicle in the aqueous environment of the cytosol (Chavrier and Goud, 1999).

The ability to travel regularly from the membrane to the cytosol and between the GTP- and GDP-bound states imposes temporal and spatial regulation of the Rab proteins binding to soluble factors that act as effectors and that transduce the signal inside the cells.

Many established, or putative, Rabs effectors and regulators have been identified and characterised. Recent evidence demonstrate that Rab effectors are not randomly distributed on the organelle but are clustered in distinct functional domains indicating that Rab GTPases and their effectors are primary determinants of compartmental specificity in the organelle of eukaryotic cells (Zerial M. & McBride H., 2001).

After internalisation from most, if not all, entry routes, cell surface molecules, including receptors, are delivered to peripheral early endosomes. The small GTPase Rab5 is one of the key regulators of this early endocytic traffic and is among the best studied Rab proteins to date (Cavalli et al., 2001).

The identification of interacting molecules has revealed the extraordinary complexity of the machinery downstream of Rab5 and indicates that Rab5 effectors and regulators function in a cooperative fashion. **(Figure 8)**

The first Rab5 effector identified and found to be essential for early endosomes fusion was Rabaptin-5 (Stenmark et al., 1995). Rabaptin-5 forms a complex with another protein, Rabex-5, which catalyses nucleotide exchange on Rab5 (Horiuchi et al., 1997). Upon activation of Rab5 by Rabex-5, the Rababptin-5/Rabex-5 complex induces its own membrane recruitment through Rabaptin-5. This complex interaction represents a positive feedback loop that counteracts GTP hydrolysis and is thought to

create a microenvironment that is enriched in active Rab5 on the membrane where other Rab5 interactors are recruited (Rybin et al., 1996).

Another important effector for Rab5 is the large coiled-coil protein EEA1 that mediates tethering/docking of early endosomes. EEA1 contains two structural elements that are essential for its targeting to the early endosome membrane. One is the FYVE finger domain, a zinc finger that specifically binds to phosphatidylinositol-3-phosphate [PtdInsP (Miller et al.)], and the other is a Rab5-binding site located immediately upstream of the FYVE finger (Stenmark and Aasland, 1999; Lawe et al., 2000; Simonsen et al., 1998). Rab5 itself interacts directly with two types of PI(Miller et al.)Ks. The first is p85a/p110b (Vanhaesebroeck et al., 1997), the second PI(Miller et al.)K is hVPS34/p150, a kinase that preferentially phosphorylates phosphatidylinositol to PtdIns(Miller et al.)P, which is necessary for the recruitment of FYVE finger proteins on the early endosome (Christoforidis, 1999; Schu, 1993; Burd and Emr, 1998).

This represents a second positive feedback loop based on the cooperativity between effectors. In this case Rab5 interacts with the hVPS34/p150 phosphatidylinositol-3-OH kinase, thus coupling PtdIns3P production to Rab5 localisation. The concomitant presence of Rab5 and PtdIns3P allows the recruitment of EEA1 and Rabenosyn-5, another FYVE finger Rab5 effector that functions in endosomes docking and fusion (Nielsen et al., 2000).

Rab5 machinery can be viewed as a modular system in which Rab5 regulators and effectors are clustered in spatial domains of action at the plasma membrane in order to generate a local amplification of active Rab5 (Zerial and McBride, 2001). However, recycling of Rab5 to the GDP-bound state is essential for normal trafficking, as the expression of GTPase-deficient Rab5 leads to the formation of giant early endosomes (Bucci et al., 1992). Thus, the activity of Rab5 GEFs and GAPs must be tightly coordinated for the maintenance of proper trafficking.

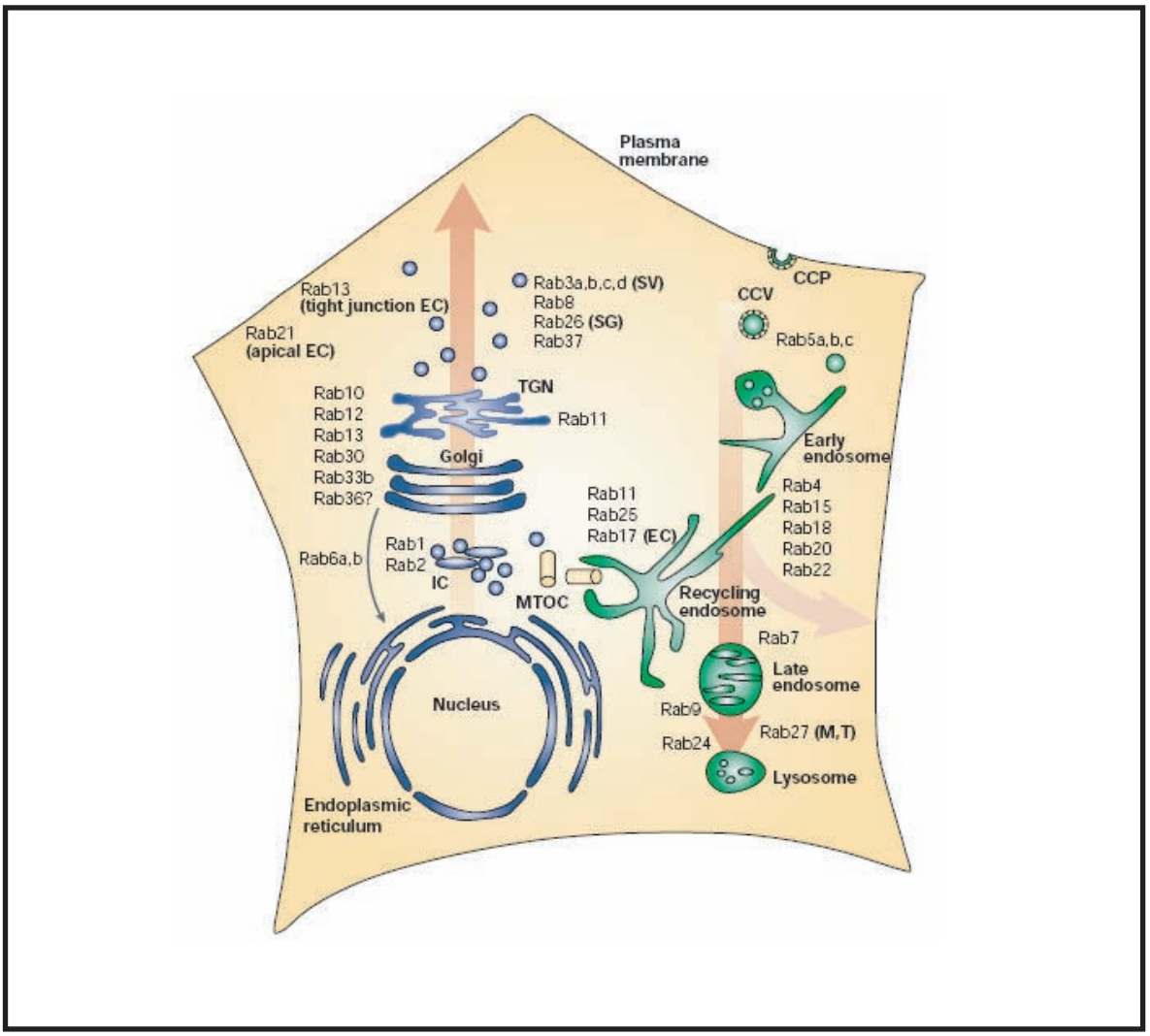



**Figure 7.** ***Map of intracellular localization of Rab proteins.***
Summarizes the intracellular localization of Rab proteins in mammalian cells. Some proteins are cell- (for example, Rab3a in neurons) or tissue-specific (for example, Rab17 in epithelia) or show cell-type-specific localization (for example, Rab13 in tight junctions). (CCV, clathrin-coated vesicle; CCP, clathrin-coated pit; EC, epithelial cells; IC, ER–Golgi intermediate compartment; M, melanosomes; MTOC, microtubule-organizing centre; SG, secretory granules; SV, synaptic vesicles; T, T-cell granules; TGN, trans-Golgi nextwork.). (Zerial & McBride, 2001)

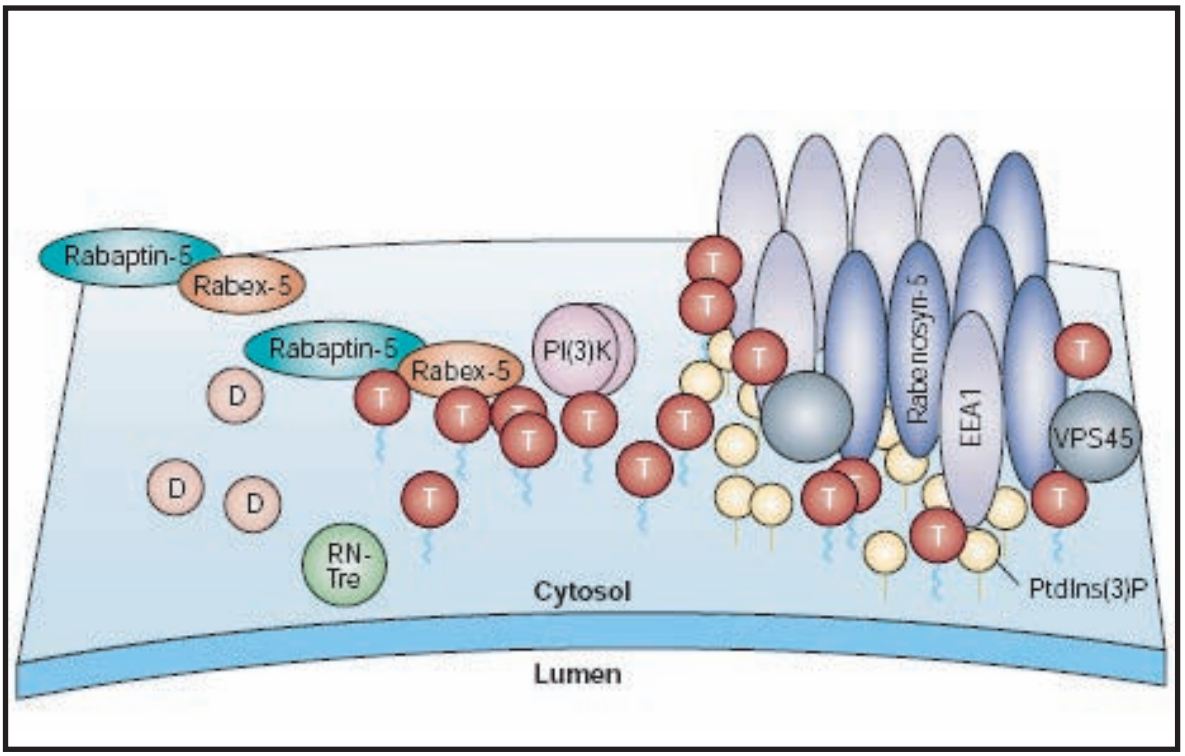



**Figure 8.** ***Rab5 effectors cluster in a Rab5 domain***

The complex network of Rab5 regulators and effectors involves positive feedback loops and, according to the model presented in the figure, is designed to generate a local amplification of active Rab5 and the clustered

recruitment of Rab5 effectors on the early endosome membrane.

Rab5-GTP (T in the figure) is unstable on the early endosome where it undergoes continuous cycles of GTP hydrolysis (Rab-GDP is shown as D in the figure), catalysed by RN-Tre33 and nucleotide exchange. The first feedback loop is due to the Rabaptin-5–Rabex-5 complex that activates Rab5 through the nucleotide-exchange activity of Rabex-5 and gets recruited on the early endosome membrane through Rabaptin-5. In this case, the product of the reaction (Rab5-GTP) recruits the enzyme. A second feedback loop is due to the cooperativity between effectors.

Active Rab5 interacts with the hVPS34-p150 phosphoinositol-3-OH kinase (PI(3)K), thus coupling phosphatidylinositol-3-phosphate (PtdIns(3)P) production to Rab5 localization. The concomitant presence of Rab5 and PtdIns(3)P allows the recruitment of the Rab5 FYVE effectors early endosome antigen 1 (EEA1) and Rabenosyn- 5.

So the Rab5 machinery can be viewed as a typical modular system, in which specific biochemical interactions between Rab5 effectors and regulators as well as other endosomal proteins create spatial segregation. By regulating the assembly of a specific membrane domain, these molecules contribute to the compartmental specificity, robustness and dynamic properties of the early endosome. (Zerial & McBride, 2001)

## *Rabex5, the best characterised guanine nucleotide exchange factor (GEF) for Rab5*

Rab GEFs promotes the binding of GTP to Rab proteins, which in turn converts them to their active signalling conformation. The founding member of the Rab5 GEF family is the yeast vacuolar sorting protein Vsp9 (Burd et al., 1996). All Rab5 GEFs have in common a catalytic unit comprising a helical bundle and a Vsp9-homology domain (Delprato et al., 2004). Rabex-5 is a human ortholog of yeast Vsp9-p, and acts on the Rab5 subfamily of low molecular weight composed of Rab5, Rab21 and Rab22 (Horiuchi et al., 1997; Delprato et al., 2004). **(Figure 9)**

Most Rab5 GEFs do not function alone, but rather as a component of larger multiprotein complexes, as exemplified by the Rabaptin-5/Rabex-5 complex (McBride et al., 1996; Lippe et al., 2001).

The incorporation of GEFs and effectors within a stable protein complex has the advantage of coupling Rab activation to downstream effector function.

Lippe and co-workers demonstrated that Rabaptin-5 and Rabex-5 functionally cooperate, but this synergy is conditional upon complex formation.

Rabaptin-5 stimulated the basal GEF activity of Rabex-5 threefold in the complex. This may result from an actual increase of Rabex-5 nucleotide exchange activity upon binding to Rabaptin-5. Alternatively, it may be that Rabaptin-5 stabilises Rabex-5 in its active folded state. This latter possibility is interesting because it suggests that Rabaptin-5 may be a chaperone for the exchange factor. Irrespective of the mechanism of this synergy, it is clear that Rabaptin-5 alone is not functional and that Rabaptin-5 and Rabex-5 mutually benefit from their association. Although Rabaptin-5 directly interacts with Rab5-GTP in two-hybrid or biochemical assays (Stenmark *et al.*, 1995), it can only be efficiently recruited onto endosomes when associated to Rabex-5.

Thus, the association of Rabaptin-5 with Rabex-5 has an important impact on Rab5 activation, effector recruitment, and function (Lippe et al., 2001).

The role of Rabex-5 in endosome fusion is subjected to additional regulation by ubiquitin-dependent modifications. As it is for the yeast Vsp9-p, Rabex-5

also binds to monoubiquitin and undergoes covalent ubiquitination (Lee et al., 2006; Mattera et al., 2006; Penengo et al., 2006).
The precise role of Rabex-5 ubiquitination remains to be elucidated but strongly suggests the necessity of a complex regulation for this GEF in the coordination of the intracellular transport steps.

## *RN-tre acts as a Rab5-GAP and concurrently as a Rab5 effector*

GTPase Activating Factors (GAPs) stimulate GTP hydrolysis and induce Rab proteins inactivation. Thus, GAPs are thought to control the lifetime of the activated state of the Rabs (Rybin et al., 1996).
Like Ras GAPs, Rab GAPs promote Rab GTPase hydrolysis by properly aligning a catalytically important glutamine residue and inserting a so called "argine finger residue" directly onto the Rab active site. This stabilises the hydrolysis reaction's transition state (Scheffzeket al., 1998; Rak et al., 2000). Replacement of this glutamine with leucine slows the rate of GTP hydrolysis, thereby locking Rabs into an active conformation (Scheffzeket al., 1998).
RN-tre was originally identified as a binding partner for the EGFR substrate Eps8; RN-tre specifically binds to the SH3 domain of Eps8 through its C-terminus (Matoskova et al., 1996). The N-terminal region of RN-tre contains a Rab family GAP homology domain, the Tre2/Bub2/Cdc16 domain (TBC or TrH), and was shown to function as a Gap for Rab5 (Lanzetti et al., 2000). **(Figure 9)**
Owing to its TrH domain activity, overexpression of RN-tre resulted in severe impairment of transferring receptor (TfR) internalisation. The endocytosis of TfR is a constitutive process, whereas other receptors, like epidermal growth factor receptor (EGFR), are internalised upon ligand engagement. Overexpression of RN-tre in Hela cells also inhibited EGFR endocytosis; thus, RN-tre regulates both constitutive and ligand-dependent endocytosis but the latter activity required the interaction between RN-tre and Eps8 (Lanzetti et al., 2000).The GAP activity of RN-tre can be finely regulated by post-traslational modification.

Initial evidence is represented by the downmodulation of the Rab-GAP activity in RN-tre immunoprecipitated from cells exposed to EGF. RN-tre is phosphorylated on serine after EGF stimulation within a time-frame similar to that of the doownregulation of its GAP activity (Lanzetti et al., 2000).

RN-tre phosphorylation status can also be modulated by Cdk activity at the onset of mitosis (Lanzetti et al., 2007). Performing in vitro GAP assays it has been demonstrated that RN-tre immunoprecipitated from mitotic cells displayed increase levels of Rab5-GAP activity compared with RN-tre derived from interphase cells (Lanzetti et al., 2007). Thus, RN-tre GAP activity can be downmodulated or upregulated depending on the specific phospho-serine status of the protein.

RN-tre also has a well documented function in actin cytoskeleton remodelling (Lanzetti et al., 2004). It has been demonstrated that Rab5 is indispensable for a form of RTK-induced actin remodelling, called circular ruffling. Rab5 signals to the actin cytoskeleton through RN-tre, which interacts with both F-actin and actinin-4, an F-actin bundling protein.

In the process of actin remodelling RN-tre has the dual function of Rab5-GAP and Rab5 effector. Indeed, it has been proposed that RN-tre establishes a three-pronged connection with Rab5, F-actin and actinin-4 and this may aid crosslinking of actin fibres into actin networks at the plasma membrane (Lanzetti et al., 2004).

In our recent work, we identified RN-tre as a novel binding partner of Cdc14A (Lanzetti et al., 2007).

Several lines of evidence suggest that RN-tre is a physiological substrate of Cdc14A. RNtre is modified by phosphorylation, which can be reversed by Cdc14A activity *in vitro* as well as *in vivo. Notably,* phosphorylation of RN-tre appears to be necessary for efficient binding to Cdc14A. Finally, the complex Cdc14A/RN-tre can be disrupted *in vitro* by sodium tungstate, an inhibitor of Cdc14A that binds in the catalytic pocket.

We reported a cell cycle-dependent phosphorylation of RN-tre, raising the possibility that this Rab-GAP could have an unexpected function during the unperturbed cell cycle. Although RN-tre appears to be weakly phosphorylated in cells arrested at G1/S and in S phase, the protein is present in a hyperphosphorylated state during mitosis. As cells exit mitosis, RN-tre phosphorylation rapidly disappears with a kinetics that is consistent

with the timing of human Cdc14A activation previously reported (Kaiser et al., 2002). These observations further imply that RN-tre phosphorylation is highly dynamic and balanced by opposing forces of kinase and phosphatase activities.

The physiological role of RN-tre/Cdc14A interaction remains unclear. However, the ability of ectopic Cdc14A and RN-tre to form a complex at the centrosome during interphase suggests that RN-tre could have a function at this site, which may be regulated by Cdc14A.

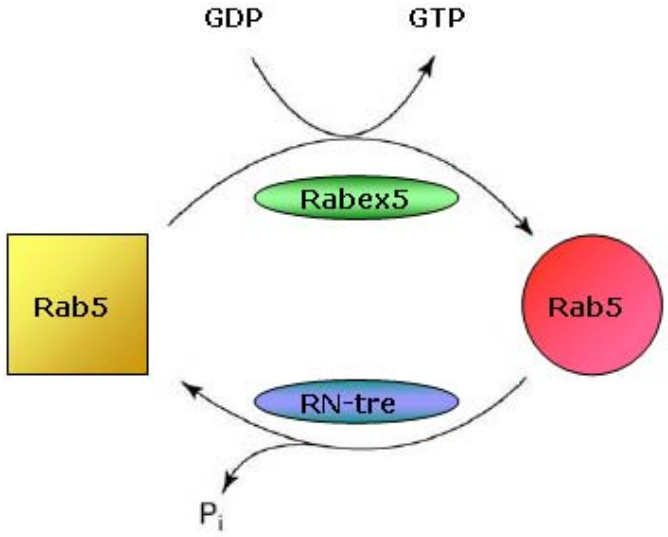
Interestingly, RN-tre contains a TBC homology domain present in the yeast proteins Bub2 and Cdc16. These proteins act as GAP proteins for the GTPases Tem1 and Spg1, which are the upstream components in the MEN and the septation initiation network signalling pathways, respectively.

Intriguingly, in budding yeast, Cdc14 interacts with the Bub2/Bfa1 two-component GAP, and Cdc14-induced dephosphorylation of the complex in late anaphase is thought to modulate its GAP activity toward Tem1 (Pereira et al., 2002). Our biochemical analysis provided initial evidence that phosphorylation may modulate the catalytic Rab5-GAP activity of RN-tre suggesting that a similar scenario involving Cdc14A and RN-tre may exist in human cells.

To date, only homologues of the downstream MEN components Cdc14 and the Dbf2-Mob1 kinase complex have been identified in humans (Stegmeier et al., 2004).

Whether RN-tre and Rab5 (or other putative Rab substrates of RN-tre) act as the functional equivalent of Bub2/Bfa1 and Tem1 in mitotic regulation is an intriguing possibility that warrants further investigation. Nevertheless, this study has provided another link between the cell cycle machinery and a protein involved in endocytic pathway.

A

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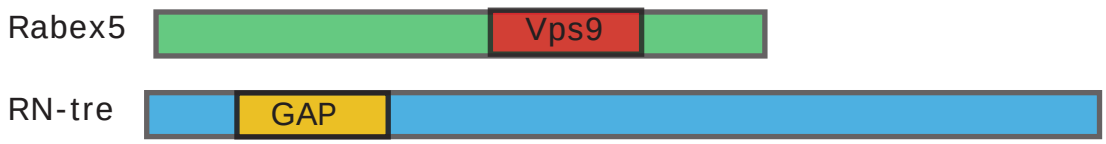



**Figure 9.** ***Structure and function of Rab5 regulators.***
A) Rabex-5 is a GEF that catalyzes nucleotide exchange of Rab5, thus promoting the active, GTP-bound form of Rab5. RN-tre is GTPase Activating Factor (GAP) and stimulates GTP hydrolysis inducing Rab5 inactivation.
B) Rabex-5, as a GEF, contains a catalytic Vps9-homology domain at the C-terminus. The N-terminal region of RN-tre contains a Rab family GAP homology domain

## *The Kinesin motor protein KIF3A*

Our research underlined the requirement of the kinesin KIF3A in the Rab5-based pathway that regulates centrosome cohesion (see results).
Here we present a general overview on the main characteristics of this kinesin motor protein.
Microtubules form a dynamic and polarised cytoskeleton. In most cell types, microtubule plus ends grow dynamically out from the microtubule-organising centre (MTOC), where the minus ends are more stably tethered (Caviston & Holzbaur, 2006).
The kinesin proteins (KIFs) along with cytoplasmic dyneins are the two major superfamilies of microtubule motor proteins. In fact, they are critical in centrosome separation, spindle formation, chromosome alignment and segregation (Held, 2000; Mountain and Compton, 2001). Kinesins are an extended superfamily, with up to 45 members expressed in mammalian cells (Miki et al., 2005). The Kinesins comprise a motor domain, which binds to the microtubule in an ATP-sensitive manner, fused to a coiled-coil domain that could mediate the association with other subunits and to a cargo-binding domain. Although the motor domains share a high degree of homology, there are considerable variations in the accessory subunits and cargo binding domains (Vale, 2003). This extensive variability means that kinesins can be functionally specific in the cell, specialised for the transport of individual cargos.

Most kinesin family motors move towards the plus end of the microtubule and are likely to be involved in trafficking events directed towards the cell periphery, such as motility from the Golgi to the plasma membrane (Vale, 2003). However, minus end-directed kinesins also contribute to intracellular trafficking events, such as the minus end directed transport of early endosomes (Bananis et al., 2000).

Kinesin-2 is one of the most ubiquitously expressed KIFs (Kondo et al., 1994; Yamazaki et al., 1995) and it has been implicated in the intracellular transport of membrane bound organelles and protein complexes in various tissue such as neurons, melanosomes and epithelial cells (Tuma et al., 1998; Jimbo et al., 2002).

Kinesin-2 is a heterotrimeric complex composed of a KIF3A/KIF3B heterodimer and KAP3 (Cole et al., 1992; Cole et al., 1993; Yamazaki et al., 1995; Wademan et al., 1996; Yamazaki et al., 1996). KIF3A and KIF3B are the microtubule based motor subunits that directly bind to each other in the absence of KAP3 (Yamazaki et al., 1995). On the other hand, KAP3 links KIF3A/KIF3B with various cargo proteins. **(Figure 10)**

Although kinesin-2 has mainly been reported to participate in intracellular transport in interphase cells, it has been shown to localise at the mitotic apparatus. In *Chlamydomonas*, the KIF3A homolog FLA10 protein is most abundant near the centrioles and the mitotic spindle during mitosis (Vashishtha et al.,1996). The sea urchin kinesin-2 homolog, kinesin II, is present transiently in the mitotic apparatus of dividing embryos (Henson et al., 1995). However, detailed functional analyses have revealed that kinesin-2 is not critical for the progression of mitosis in either *Chlamydomonas* or sea urchin embryos (Miller et al., 2005; Morri et al., 1997). The function of kinesin-2 during mitosis in mammalian cells has been recently investigated by Haraguchi and co-workers (Haraguchi et al., 2005). They found that the KIF3A/KIF3B complex is localised at the centrosomes in interphase Hela cells and at the spindle microtubule during metaphase. Conversely, KAP3 localises at the centrosomes after the cells entered prometaphase.

Furthermore, Haraguchi and co-workers showed that the expression of a dominant negative mutant of KIF3A/KIF3B, unable to form a complex with KAP3, caused abnormal spindle formation and chromosomal aneuploidy. Hence, the interaction between KIF3A/KIF3B and KAP3 may be required for spindle formation and chromosome segregation, although one cannot exclude the possibility that a protein(s) other than KAP3 also interacts in and is critical for this function.

Intriguingly, it has been reported that the tumour suppressor adenomatous polyposis coli (APC) localises to the ends of microtubules embedded in kinetochores and plays a critical role in chromosome segregation (Fodde et al., 2001; Kaplan et al., 2001). Since it had been previously found that APC is associated with KIF3A/3B via its interaction with KAP3 (Jimbo et al., 2002), APC may be one of these critical cargo proteins involved in the progression of mitosis.

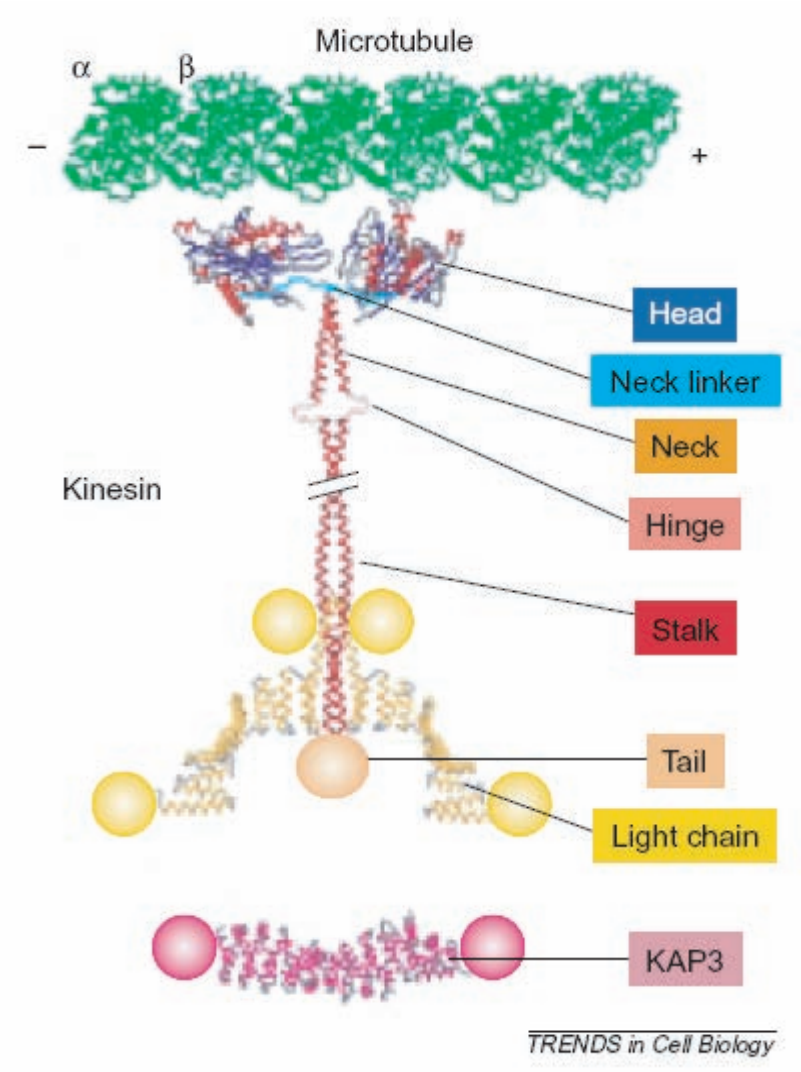



**Figure 10.** ***Kinesin domain structure and associated proteins.***
The diagram shows some known molecular structures in backbone representation (without side chains). The top structure represents a protofilament of a microtubule, the 'track' for kinesin motors, containing alternating α- and β-tubulin subunits (light and dark green, ≈450 residues each). The blue and red structure in the middle represents a dimeric kinesin heavy chain (KHC) as found in KIF5B, the conventional kinesin-I from rat brain. Each heavy chain contains a motor domain ('head', α-helices red, β-strands blue) that binds to ATP and microtubules, a neck linker (cyan) whose conformation changes during the ATPase cycle, an ?-helical neck and a stalk (red) that causes dimerization by a coiled-coil interaction. The globular tail domain (≈50 to 130 residues) is structurally not known and represented as a red sphere roughly in scale to its expected size. The C-terminal ≈320 residues dock to the cargo or scaffolding proteins of the transport vesicle – e.g. JIP, amyloid precursor protein (APP), kinectin or others. The kinesin light chain structure is not known. In the case of the heterotrimeric KIF3/kinesin-II structure, the two heavy chains are distinct (KIF3A and B) but otherwise have broadly similar domain. KAP3 (shown, magenta) is largely α-helical and contains 11 'armadillo' repeats. Each repeat contains ≈42 residues, folded into units of three α-helices. (Mandelkow, 2002)

# Results

## *Rab5 and its regulators localise at the centrosome*

The analysis of the sub-cellular localisation of a protein is a good starting point to discover its function.

In our previous work we described the retention of exogenous RN-tre at the centrosome following ectopic expression of Myc CDC14PD in U2OS cells (Lanzetti et al., 2007). Prompted by this finding we decided to analyse in more detail the distribution of RN-tre and its molecular partners Rab5 and Rabex5, thus we detected a previously unrecognised localisation of these proteins at the centrosome.

## RN-tre and GFP-Rabex5 are novel components of the centrosome

RN-tre displays multiple cell localisation, being prominent at the plasma membrane, but also detectable in association with intracellular membranes, and in the cytosol (Lanzetti et al.,2004; Lanzetti et al.,2000). By performing anti-RN-tre immunostaining in cytosol-depleted U2OS osteosarcoma cells, we detected the co-localisation of RN-tre with $\gamma$-tubulin at the centrosome.

Moreover, double labelling with anti-RN-tre and anti-centrin antibodies showed localisation of RN-tre on the side of centrin-positive centrioles. The specificity of the antibody used in this study is confirmed by the absence of specific staining in U2OS cells depleted for RN-tre (RN-tre KO).

**(Figure 11A)**

In addiction we observed that the recruitment of RN-tre at the centrosome wasn't affected by the disruption of the microtubule network, performed by

nocodazole treatment of U2OS cells (data not shown). Such nocodazole resistant centrosomal localisation was previously reported for integral pericentriolar protein such as γ-tubulin or pericentrin.

To identify the determinants responsible for the association of RN-tre with the centrosome, we engineered RN-tre mutants, fused to the green fluorescent protein (GFP), and analysed their co-localisation with γ-tubulin. **(Figure 11B)**

As expected, ectopically expressed GFP-RN-tre localised to the centrosome, a property maintained by its isolated C-terminal fragment (GFP-RN-tre aa 574-828).

Conversely, the GAP domain-containing, N-terminal fragment of RN-tre (GFP-RN-tre aa 1-395) did not co-localise with γ-tubulin. Therefore RN-tre is targeted to the centrosome through its C-terminus. **(Figure 11C, see also BOX1)**

We next examined the localisation of GFP Rabex5, and we found that this protein is also present at the centrosome, where it co-localises with the centrosomal markers γ-tubulin and centrin. **(Figure 12A)**

The lack of an antibody that recognised Rabex5 in immunofluorescence prevented the study of the localisation of the endogenous protein; of note, when the GFP Rabex5 was expressed at low levels its major localisation was at the centrosome.

To further investigate the presence of RN-tre and Rabex5 we analysed purified centrosomal preparation by immunoblot. Both RN-tre and Rabex5 co-fractionated with centrosomal markers such as Nek2 and γ-tubulin in sucrose gradient fractions. This finding suggests that RN-tre and Rabex5 are real novel components of the centrosome. **(Figure 12B)**

Interestingly a limited amount of Rab5 was also detectable in purified centrosomes. Transferrin receptor, which is present both on early endosomes and on the pericentriolar recycling endosomes (Mellman, 1996) like Rab5 does, was not detectable, indicating that contaminations by such vesicular compartments can be excluded. **(Figure 12B)**

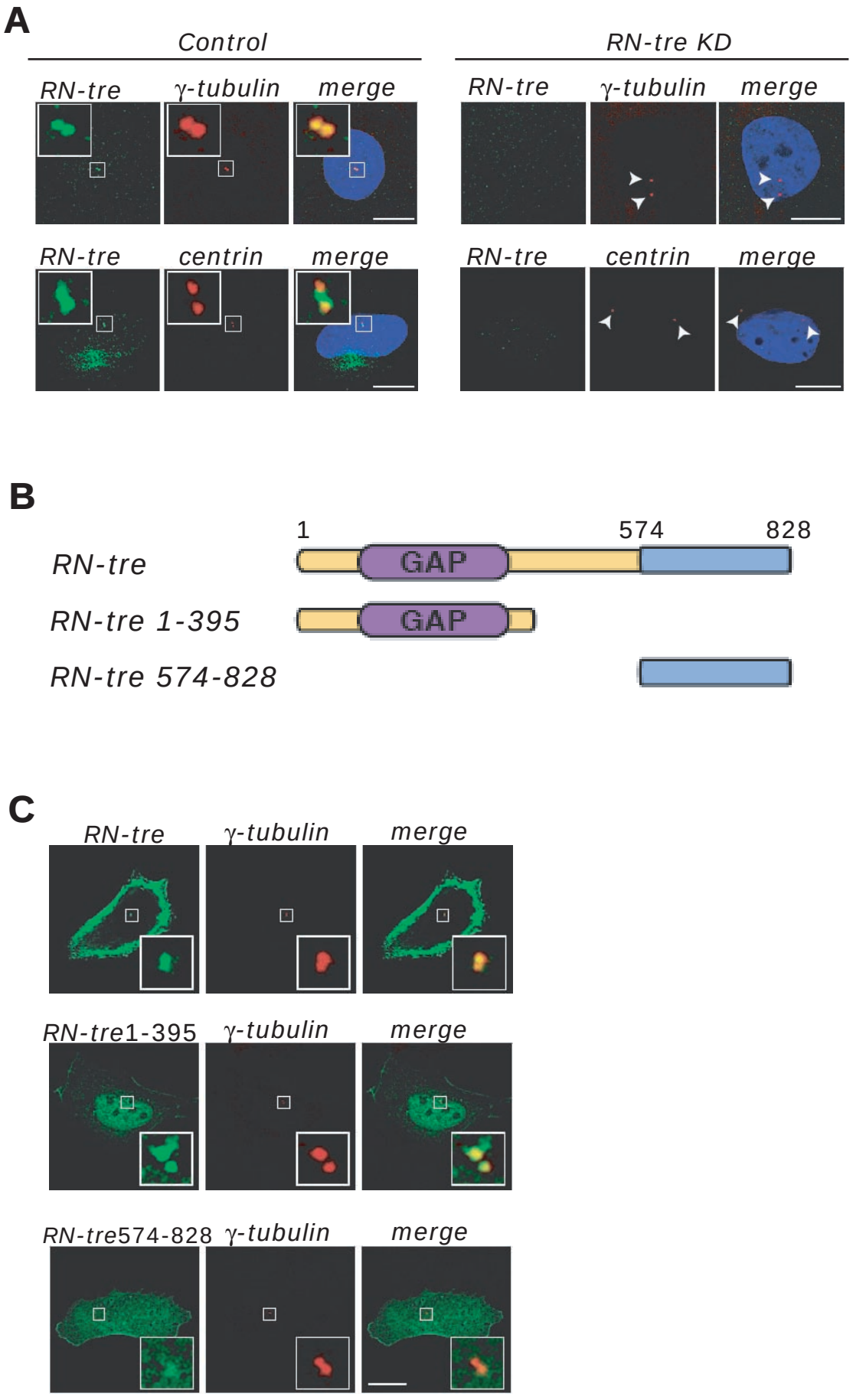

A
Control
RN-tre KD
RN-tre
γ-tubulin
merge
RN-tre
centrin
merge
B
1
574
828
RN-tre
GAP
RN-tre 1-395
GAP
RN-tre 574-828
C
RN-tre
γ-tubulin
merge
RN-tre1-395
γ-tubulin
merge
RN-tre574-828
γ-tubulin
merge

Figure 11

**Figure 11*. RN-tre is targeted to the centrosome through its C-terminus.***

A) Confocal analysis of U2OS cells silenced with control oligos (Con.) or with siRNA oligos for RN-tre (RN-tre-KD). Cells were subjected to cytoplasmic extraction and stained for RN-tre (green) and either for γ-tubulin or centrin (red) as indicated. Merged images are also shown (blue, DAPI). Insets (in Con.) show enlargements of the centrosomal region. Arrowheads (in RN-tre-KD) point to split centrosomes/centrioles (> 2 μm apart). Bar, in this and all subsequent figures (unless otherwise specified), 10 μm.

B) Schematic representation of the different GFP-tagged plasmid used to identified the domain responsible for RN-tre localization at the centrosome.

C) Confocal analysis of U2OS cells, transfected with the indicated (left) GFP-tagged plasmids (green), stained for γ-tubulin (red). Merged images are also shown. Centrosomal regions are boxed and magnified in the insets.

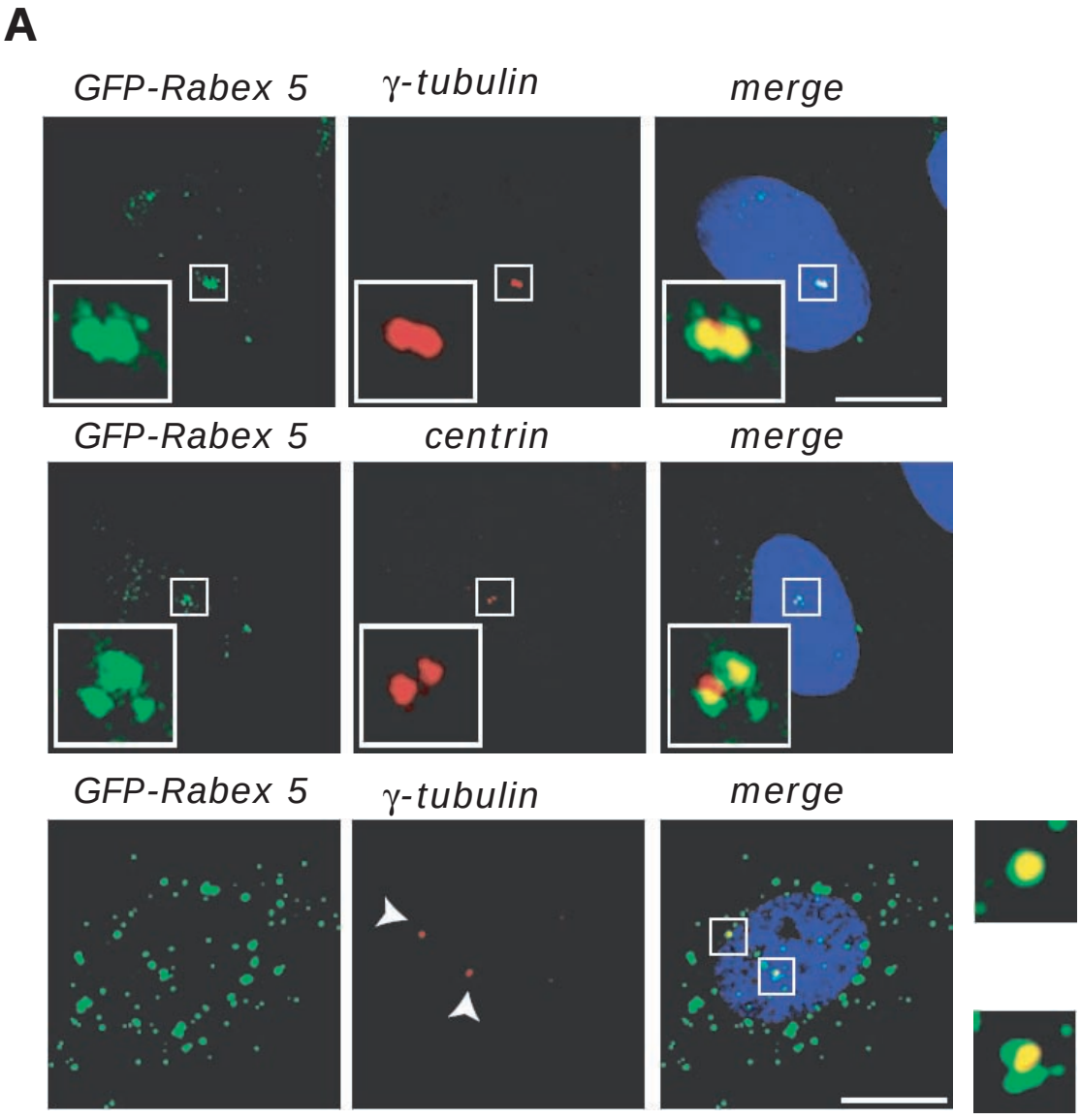

A
GFP-Rabex 5
γ-tubulin
merge
GFP-Rabex 5
centrin
merge
GFP-Rabex 5
γ-tubulin
merge
B
Sucrose (%)
70
45
WCE 1 2 3 4 5 6 7 8 9 10 11 12 13 14
RN-tre
Rabex 5
Rab 5
Tfr
Nek2
γ-tubulin

Figure 12

**Figure 12*. RN-tre and Rabex-5 are novel component of the centrosome.***

A) Confocal analysis of U2OS cells, expressing GFP-Rabex-5 (green), stained for either γ-tubulin or centrin (red,) as indicated. Merged images are also shown (blue, DAPI). The centrosomal regions are magnified in the insets. Bottom, split cetrosomes (arrowheads) are boxed in, and magnified in the insets on the right.

B) Centrosomes purified from human KE37 cells, were analyzed in IB as indicated (Nek2 and γ-tubulin are centrosomal markers). WCE, whole cell extract (~5-fold more total proteins were loaded in the WCE lane, compared to centrosome fraction 8).

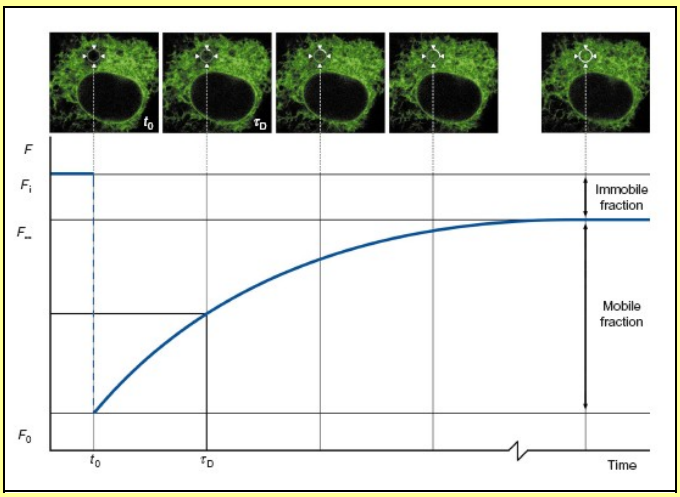
# BOX1. Studying RN-tre turnover at the centrosome

New and updated fluorescence imaging methods allow the examination of the localization and the kinetic behaviour of GFP tagged protein.

To establish the living cell dynamics of RN-tre associated with the centrosome FRAP (Fluorescence Recovery After Photobleaching) experiments were performed.

In FRAP experiments fluorescent molecules are irreversibly photobleached in a small area of the cell by a high-powered focused laser beam. Subsequent duffusion of surrounding non-bleached fluorescent molecules into the bleached area leads to a recovery of fluorescence, which is recorded at low laser power.

FRAP experiments provide information about motility of a fluorescent molecule in a defined compartment. Two parameters can be deduced from FRAP: the mobile fraction of fluorescent molecule and the rate of mobility, which is related to the characteristic diffusion time $t_{D.}$ (Reits and Neefjes, 2001).




**Fluorescence recovery after photobleaching (FRAP).** When a region in the fluorescent area (here the endoplasmic reticulum) is bleached at time t0 the fluorescence decreases from the initial fluorescence Fi to F0. The fluorescence recovers over time by diffusion until it has fully recovered (F∞). The characteristic diffusion time tD indicates the time at which half of the fluorescence has recovered. The mobile fraction can be calculated by comparing the fluorescence in the bleached region after full recovery (F∞) with that before bleaching (Fi) and just after bleaching (F0)

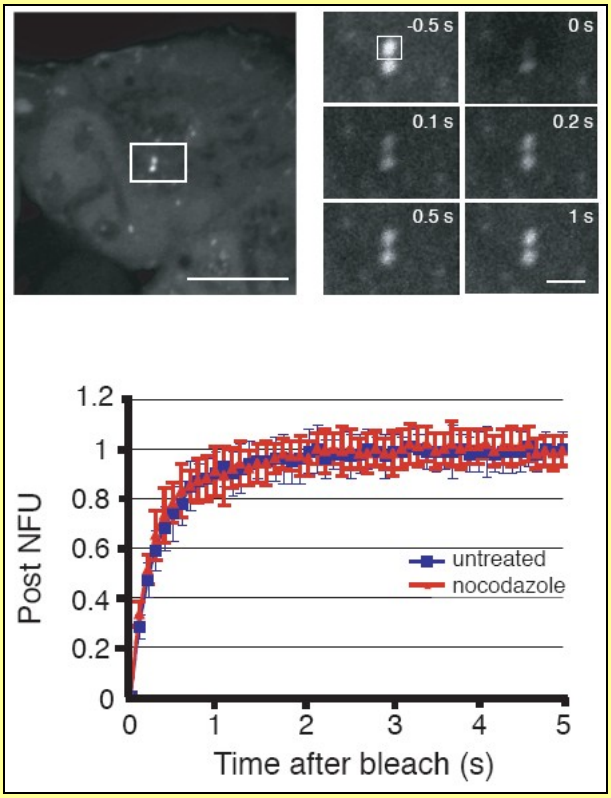
In U2OS cells expressing GFP-RN-tre 574-828 a square centred on one centrosome were bleached and recovery of the fluorescence was imaged over time. The recovery of GFP fluorescence was extremely rapid, ($t_{1/2}$ ~0.45s) and reached the plateau after ~1s. As it was expected by the results obtained on fixed cells, microtubule depolymerization had no effect on the time and % of the recruitment of GFP-RN-tre 574-828 at the centrosome.

The centrosome assembly in mammalian cells has been extensively studied; however, mechanisms involved in recruiting centrosomal proteins are poorly understood.

FRAP studies on the dynamics of regulatory molecules, such as Nek2 (Hames et al., 2005) and Aurora A (Stenoien et al., 2003) indicate rapidly exchanges in and out of the centrosome ($t_{1/2}$~3s). Conversely the structural components of the centrosome, α and γ-tubulin (Khodjakov and Rieder, 1999; Stenoien et al., 2003) are relatively immobile.

These observations fit a model in which the centrosome is composed of a relatively stable scaffold to which regulatory molecules associate in a transient manner.

Considering it extremely rapid turnover, RN-tre as the characteristics of a regulatory centrosomal element.




**FRAP experiment on GFP-RN-tre574-828.**

A representative cell expressing GFP-RN-tre574-828 and the images collected at various times during the FRAP experiment are shown(bar, 2 μm). Only one of the duplicated centrosomes was bleached (small box on the right). Note also that fluorescent signal at the non-bleached centrosome decreases at t0, underscoring the highly mobile nature of GFP-RN-tre574-828.

Quantification of FRAP results in cells pre-treated with nocodazole (5 μg/ml, 2-4 h before FRAP) or untreated. Mean values ± SD (n=15) are shown. Recovery of centrosome associated fluorescence was 86 ± 11% of initial fluorescent intensity (not shown).

## Rab5 localises at the centrosome in a cell cycle dependent manner

Since a limited amount of Rab5 that co-fractionated with the centrosomal markers Nek2 and γ-tubulin, we performed a confocal analysis of the localisation of this protein at the centrosomes.

In agreement with previous observations (Nielsen et al., 1999), we visualised yellow fluorescent protein (YFP)-tagged Rab5 on endosomes and in the juxtanuclear region, adjacent to the centrosome. In particular YFP-Rab5 appeared to be on small membranous tubules (which do not correspond to Golgi membranes, Supplemental Fig. 1) embracing the γ-tubulin positive dots. **(Figure 13A)**

Similarly to ectopically expressed YFP-Rab5, also endogenous Rab5 was in close proximity to the centrosome. This localisation was maintained on duplicated centrosomes until prophase/prometaphase, but it was lost at metaphase and during anaphase. When the cell reached cytokinesis Rab5-positive staining reappear close to the centrosome. **(Figure 13B)**

The anti-Rab5 antibody employed in the analysis recognises Rab5A as confirmed by the absence of any specific staining in the cells silenced for Rab5A.

As with RN-tre, we analysed the distribution of Rab5 after microtubule network disruption, and we saw the loss of Rab5 localisation at the centrosome in cells treated with nocodazole (data not shown); Rab5 reappeared in close proximity to the centrosome after microtubules regrowth, suggesting a functional, but not structural, role for this small GTPase in centrosome homeostasis.

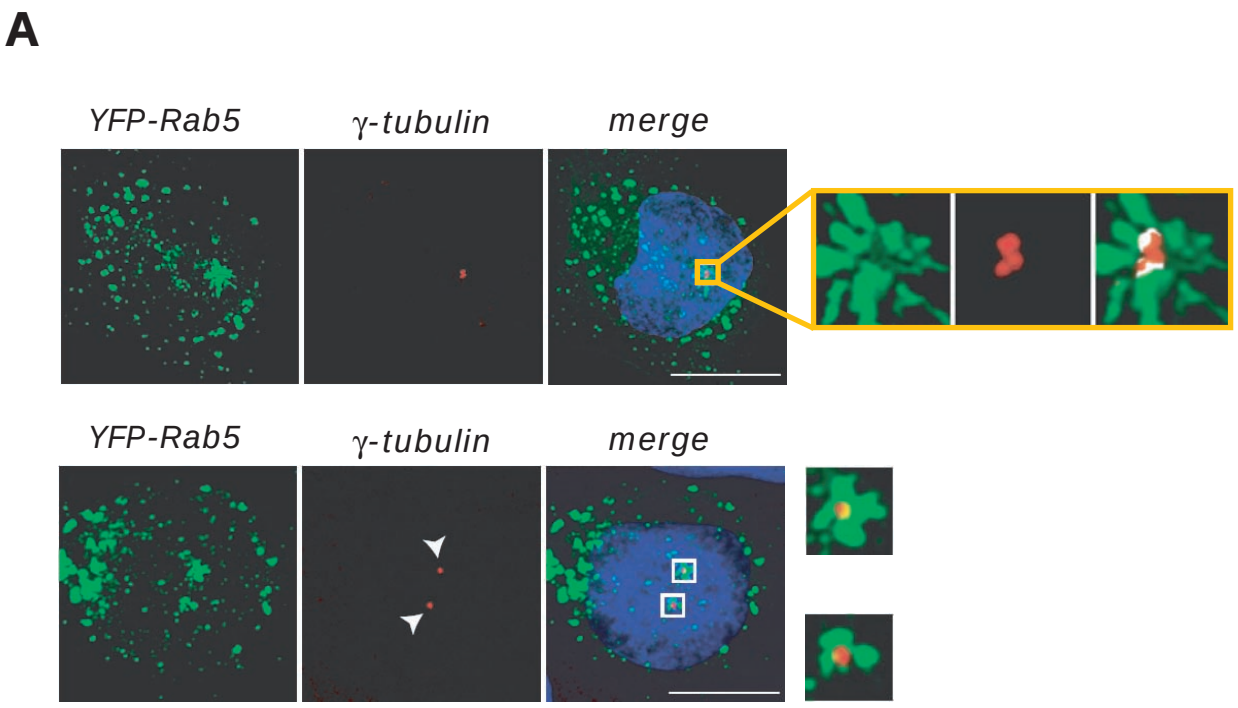

A
YFP-Rab5
γ-tubulin
merge
YFP-Rab5
γ-tubulin
merge
B
interphase
prophase
metaphase
anaphase
telophase
Rab 5-KD

Figure 13

**Figure 13.** ***Rab5 localises at the centrosome in a cell cycle dependent manner.***

A) Top: confocal analysis of U2OS cells, expressing YFP-Rab5 (green) and stained for γ-tubulin (red, top). Merged images are also shown (blue, DAPI). The centrosomal region shows duplicated centrosomes still paiered magnified in the insets. The region of overlap between YFP-Rab5 and γ-tubulin has been automatically calculated by means of the Leica Confocal Software, Version 2.61(Leica Microsystems) and appears as a white mask in the merge. Bottom: arrowheads point to split centrosomes which are boxed in and magnified on the right.

B) Confocal analysis of U2OS cells at different cell cycle stages (indicated on top) stained with anti-Rab5 (mouse anti-Rab5 clone1 Becton Dickinson, red), anti-γ-tubulin (green) and DAPI. The cell in the last panel on the right has been silenced for Rab5A (Rab5A-KD) as described in Experimental Procedures. Centrosomal region is magnified in the insets.

## *Rab5 and its regulators affect centrosomes separation*

To understand the function of Rab5 and its regulators at the centrosome we performed gain of function or loss of function experiments, overexpressing or downregulating the protein in exam. Thus, we underline the implication of Rab5, RN-tre and Rabex5 in the regulation of centrosomes cohesion.

### Excess of Rab5 causes centrosome splitting

Although Rab5 does not seem to be a major steady state component of the centrosome, its over-expression resulted in centrosome splitting in around one third of the transfected cells.

For the sake of clarity, we will use the term “centrosome splitting” to describe any separation of parental centrioles regardless of the presence or absence of pericentrioles. In particular, centrosomes, were scored as split when the distance between the γ-tubulin positive dots was greater then 2 μm according to the procedure described in (Meraldi and Nigg, 2001).

We overexpressed separately all three distinct isoforms of Rab5, (Rab5A, Rab5B and Rab5C) and it is noteworthy that ectopic expression of all the proteins induced centrosome splitting to similar extents. These findings are in agreement with previous observations, showing that the function of the three Rab5 proteins in endocytosis is redundant (Bucci et al., 1995; Huang et al., 2004) **(Figure 14A)**.

In addiction we silenced RN-tre by RNAi in U2OS cells and this resulted in a larger number of cells with split centrosomes. **(Figure 14B and C, see also Box 2)**

The increase in centrosome splitting was not attributable to an higher proportion of cells in G2/M, as revealed by FACS analysis. (Data not shown)

The overexpression of Rabex5 leaded to the same phenotype, inducing centrosome splitting. **(Figure 14D)**

Therefore, we could conclude that excess of active Rab5, achieved by overexpressing the GTPase it-self or its GEF, Rabex-5, or by silencing the GAP, RN-tre, disrupt centrosome cohesion.

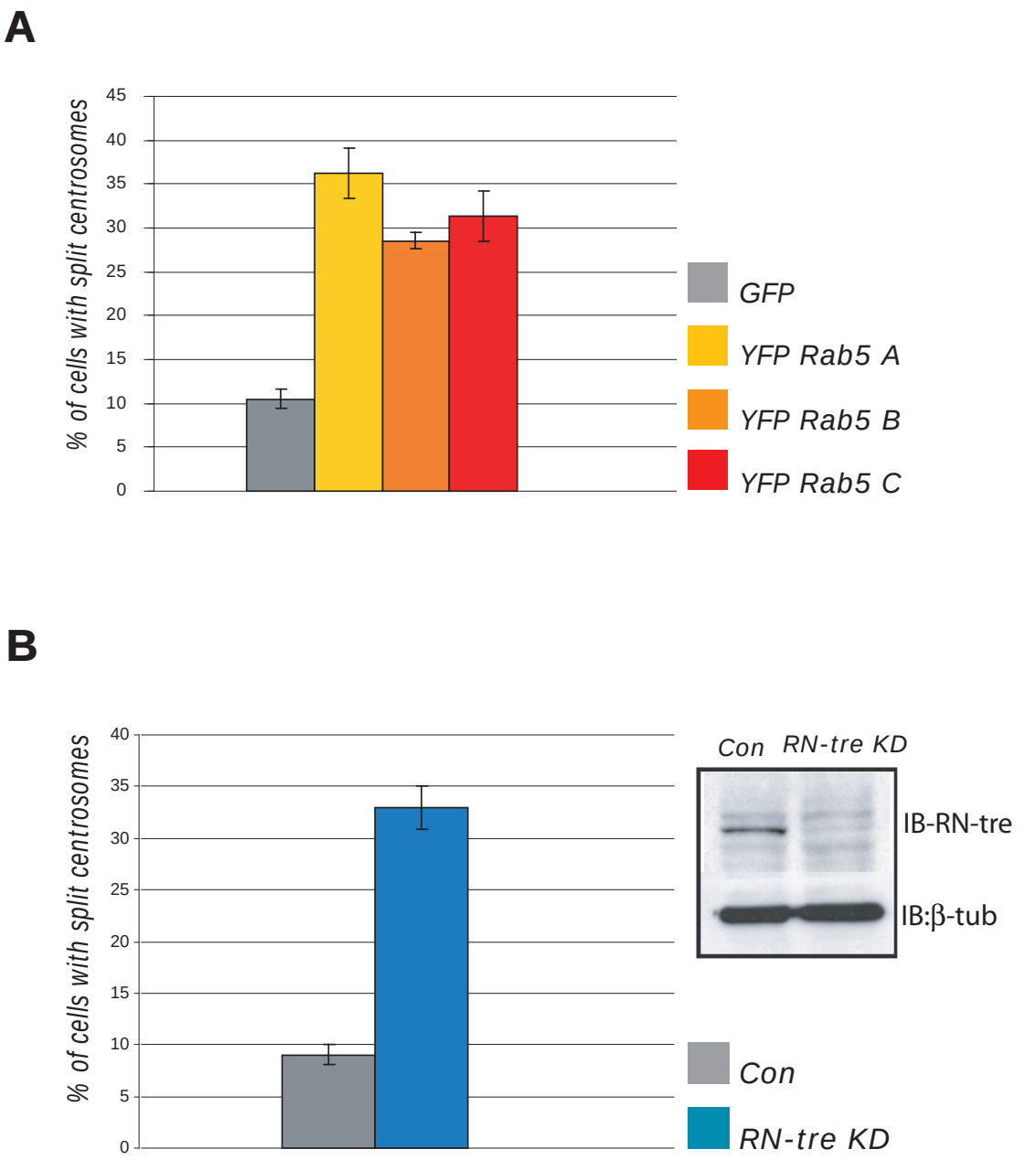

A
% of cells with split centrosomes
45
40
35
30
25
20
15
10
5
0
GFP
YFP Rab5 A
YFP Rab5 B
YFP Rab5 C
B
% of cells with split centrosomes
40
35
30
25
20
15
10
5
0
Con
RN-tre KD
IB-RN-tre
IB:β-tub
Con
RN-tre KD
C
% of cells with split centrosomes
30
25
20
15
10
5
0
GFP
GFP Rabex5

Figure 14

**Figure 14.** ***Rab5 and its regulators affect centrosome separation***

A, B, D) Bar graphs showing the percentage of cells displaying split centrosomes. Mean values (n=5, 200 cells/condition/experiment) ± standard deviation are shown (p value <0,01).

C) Total lysates (50 µg) from RN-tre-KD cells or control cells were immunoblotted (IB) as indicated. (C)

# BOX2. Functional domains of RN-tre required to maintain centrosome cohesion.

We performed ablation/reconstitution experiments, to test the ability of RN-tre mutants to rescue the centrosome splitting phenotype of the RN-tre-silenced cells. To this end, we cloned an RN-tre-silencing oligo in the pAV vector, and generated stably silenced U2OS clones. The stably silenced clone 1 (Cl.1) displayed a reduction in RN-tre protein levels of > 80% and showed split centrosomes in ~ 40% of the cells. Similar results were obtained with several other clones (not shown). Cl.1 was then reconstituted with wt RN-tre or several RN-tre mutants. Since the RN-tre mutant, RN-tre1-395, containing the GAP domain, does not localize at the centrosome, we also engineered an RN-tre1- 395 mutant harbouring the centrosomal localization region of AKAP450 (RN-tre1-395AKAP).

We found that only the re-expression of RN-tre full length was able to rescue the split centrosome phenotype in Cl.1. The GAP domain alone was unable to recover paired centrosomes, even if artificially targeted to the centrosome (F. However, the GAP activity of RN-tre, albeit not sufficient, was required for the maintenance of centrosome cohesion, as demonstrated by the lack of rescue by both an RN-tre mutant devoid of GAP activity [RN-treR150, described in (Lanzetti et al., 2000)], and the isolated C-terminal region (RN-tre547-828), lacking the GAP domain.

Collectively, these results reveal two distinct regions in RN-tre both required to maintain centrosome cohesion: the GAP domain, which regulates Rab5 activity, and the C-terminus, which could bind to centrosomal component(s).

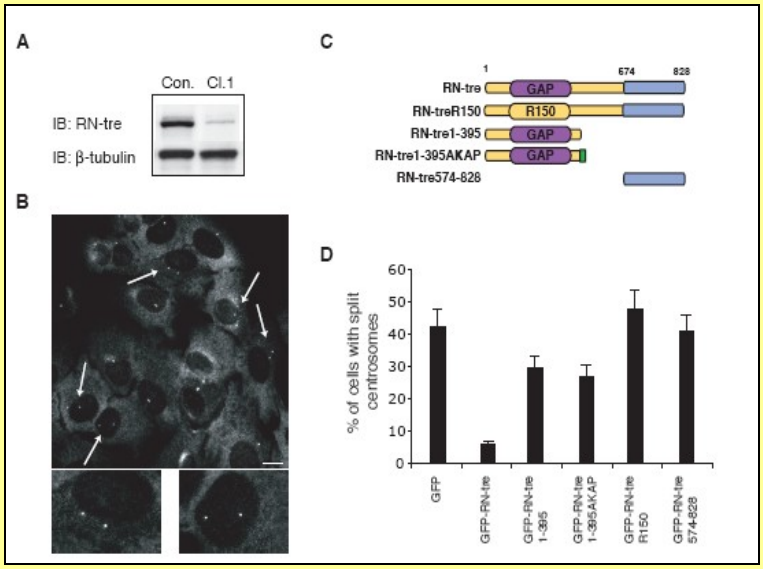



**Figure 6. Functional domains of RN-tre required to maintain centrosome cohesion.** (A) Total lysates from control and the stable RN-tre-silenced clone (Cl.1) were IB as indicated. (B) IF of clone Cl.1 with anti-γ-tubulin. Arrows point to cells with split centrosomes. (C) Scheme of RN-tre mutants. (D) Bar graph showing the percentage of cells displaying split centrosomes in RNtre-silenced Cl.1, upon transfection of the GFP-tagged plasmids indicated on bottom.

## Rab5Q79L promotes centrosome separation at G2 and centrioles splitting at the onset of mitosis

In the attempt to better characterise the Rab5-induced centrosome splitting, we generated a U2OS cell line that conditionally expressed the dominant active Rab5 mutant, Rab5Q79L. Expression of the mutant, determined by doxycycline stimulation, increased the number of H3(S10-P)-positive cells with separated centrosomes, while it did not significantly affect centrosome splitting in H3(S10-P)-negative cells. **(Figure 15)**

Rab5Q79L did not have a major effect on the distribution of cells in different parts of the cell cycle as shown by FACS analysis. **(Figure 16A)**

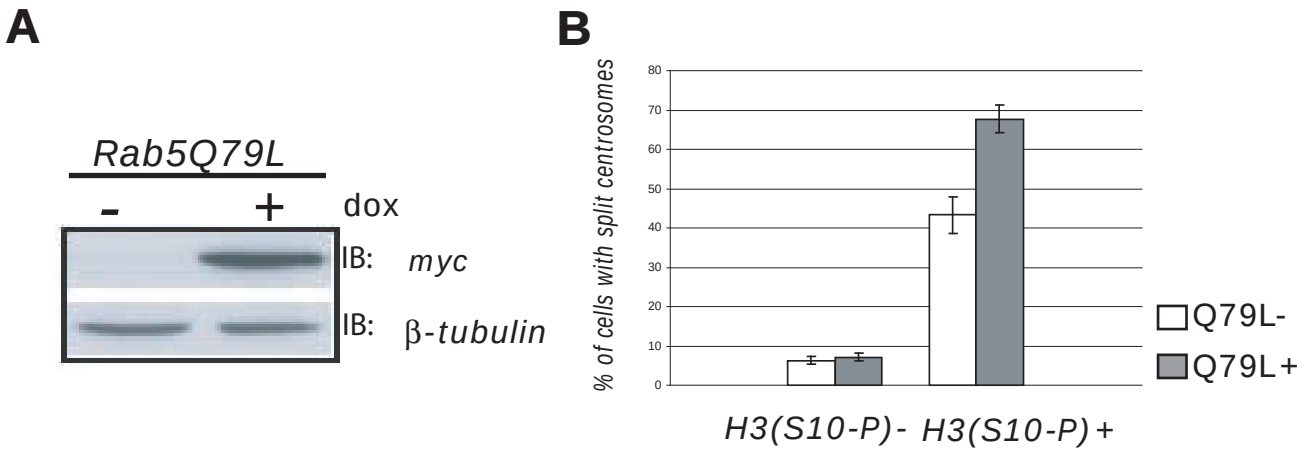
In addition, we noticed that the Rab5Q79L-expressing cells at late G2/prophase showed duplicated centrosomes, in which at least one of the two centrosomes split and the centrioles moved apart.

Moreover, these cells showed an increased frequency of aberrant mitosis, with multiple spindles (32% vs. 8% in control cells). Importantly, the same phenomenon was detected upon RN-tre silencing (RN-tre-KD) (28% aberrant mitosis). **(Figure 16B, C, D and E)**

It is likely that abnormal mitosis resulted from split sister centrioles that retained the ability to nucleate microtubules, thus generating supernumerary spindle poles.

The faculty of Rab5Q79L to induce centrioles splitting at mitosis was blocked in cells synchronised at pro-metaphase by nocodazole treatment and it was rescued upon nocodazole wash-out indicating that it requires microtubules integrity. **(Figure 16F)**

While our analysis does not exclude that additional defects could be at play at cytokinesis, it allows us to propose that activation of Rab5 triggers centrosome separation at G2.


A
Rab5Q79L
-
+
dox
IB: myc
IB: β-tubulin
B
% of cells with split centrosomes
80
70
60
50
40
30
20
10
0
Q79L-
Q79L+
H3(S10-P)-
H3(S10-P)+

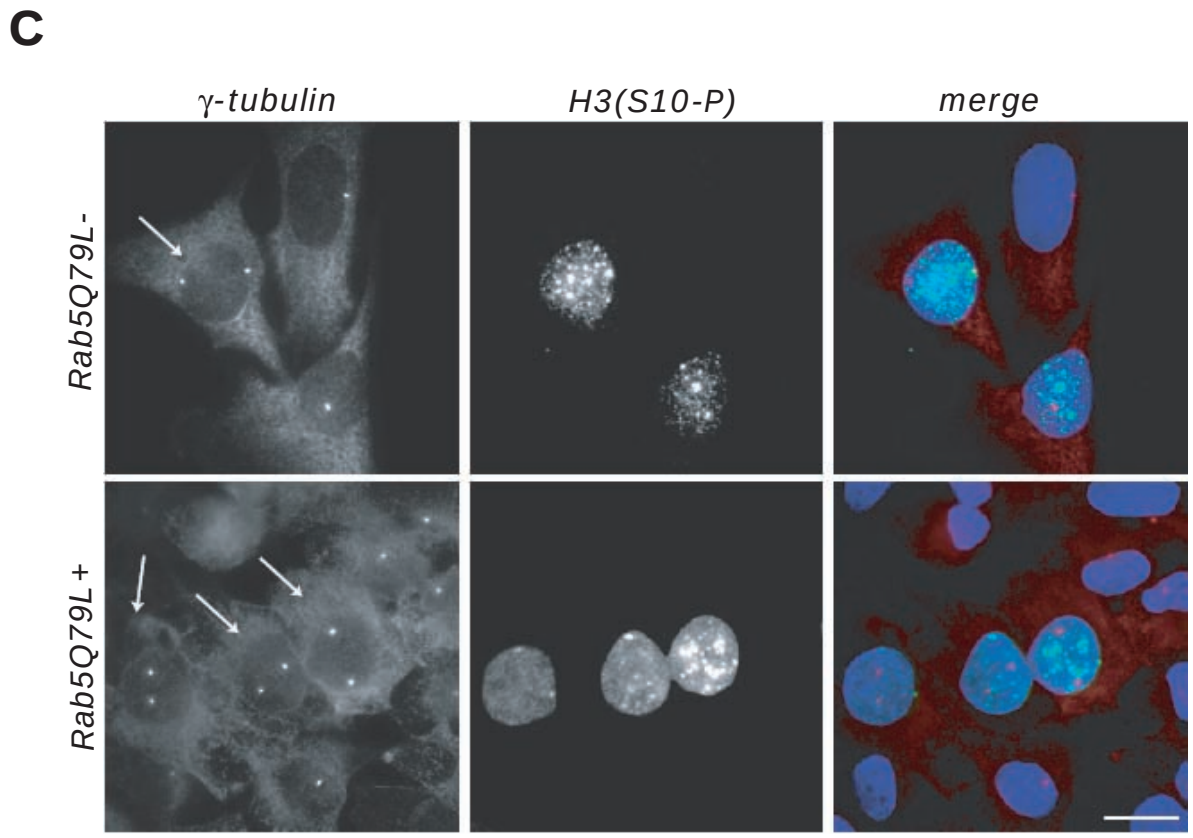

C
γ-tubulin
H3(S10-P)
merge
Rab5Q79L-
Rab5Q79L+

Figure 15

**Figure 15.** ***Rab5Q79L promotes centrosome separation during G2.*** The U2OS cell line conditionally expressing the dominant active Rab5 mutant, Rab5Q79L was treated for 24h with doxycycline (+) or left untreated.

A) Total cellular lysates (50 µg) IB as indicated (myc, detection of myc-tagged Rab5Q79L).

B) Cells were stained with anti-H3(S10-P) and anti-γ- tubulin antibodies. The bar graph shows the percentage of H3(S10-P)-negative, or –positive cells displaying split centrosomes. Mean values ± SD are shown. (n=4, 200 cells/condition/experiment; p value <0,01).

C) Representative immunofluorescence pictures of the experiments in B.

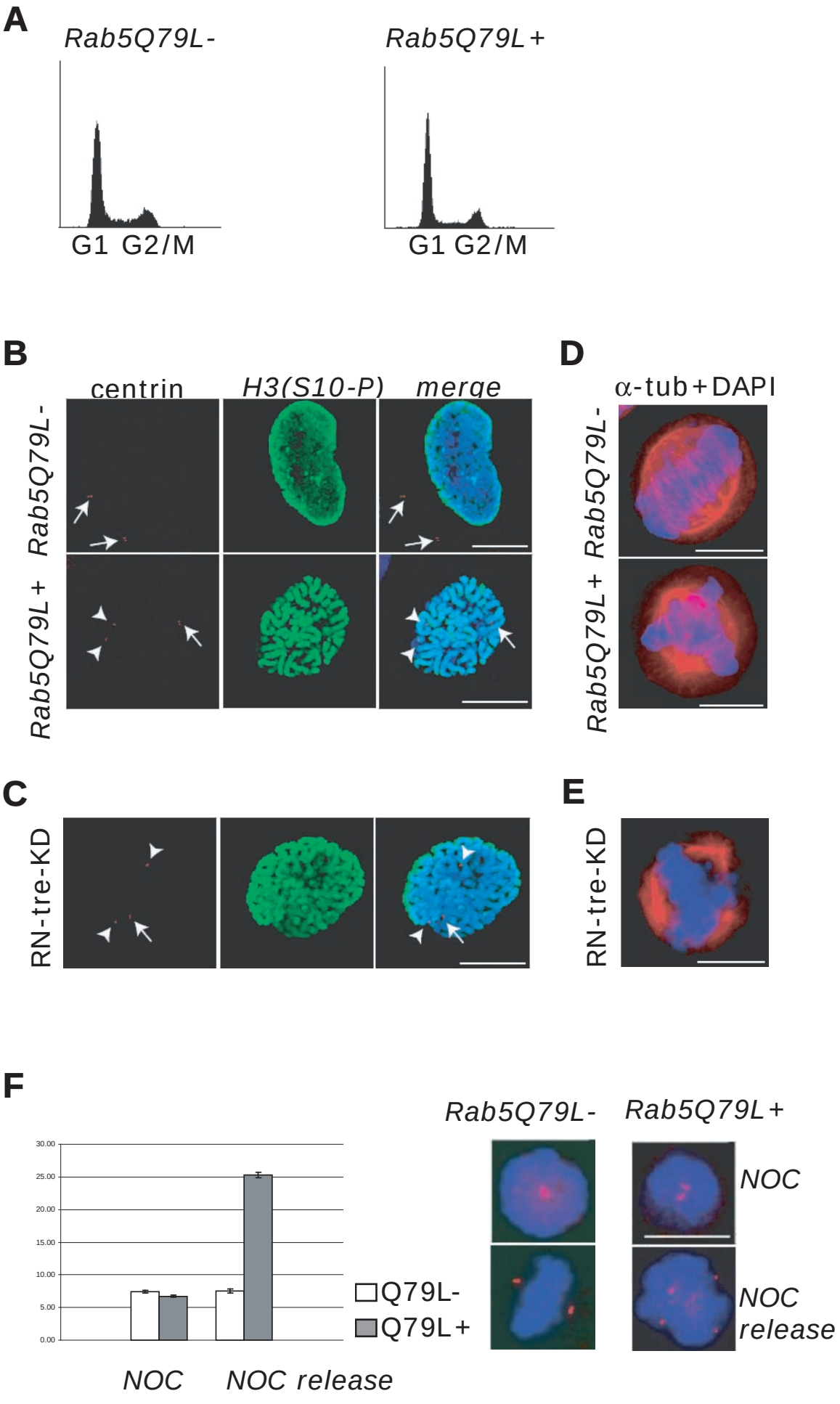

A
Rab5Q79L-
Rab5Q79L+
G1 G2/M
G1 G2/M
B
centrin
H3(S10-P)
merge
Rab5Q79L-
Rab5Q79L+
D
α-tub+DAPI
Rab5Q79L-
Rab5Q79L+
C
RN-tre-KD
E
RN-tre-KD
F
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Q79L-
Q79L+
NOC
NOC release
Rab5Q79L-
Rab5Q79L+
NOC
NOC release

Figure 16

**Figure 16.** ***Rab5Q79L causes sister centrioles splitting at the onset of mitosis.***

A) The U2OS cell line conditionally expressing the dominant active Rab5 mutant, Rab5Q79L was treated for 48h with doxycycline (Rab5Q79L+) or left untreated (Rab5Q79L-) and analyzed by FACS.

B-D) Representative confocal images of H3(S10-P) positive cells in U2OS cells stably transfected with the inducible (TET ON) Rab5Q79L, and either mock treated(Con., B top panel), or treated with doxycycline for 24 hours (Rab5Q79L, B bottom panel), or in U2OS cells silenced for RN-tre (C). Cells were stained with anti-centriņ (red), and anti-H3(S10-P) (green). Merged images are also shown (blue, DAPI). Note that in the control cell (B, top panel shown at prophase) both the duplicated centrosomes (arrows) contain two-paired centrioles that are migrating to the opposite poles of the cell; conversely, Rab5Q79L-expressing (B, bottom panel), and RN-tre-KD (C) cells (both shown at prometaphase) have one paired centrosome (arrow) and two split centrioles (arrowheads).

D-E) Immunofluorescence of mitotic cells from the same samples as in B-C are shown, stained with anti-α-tubulin antibody (red) and DAPI (blue).

F) The U2OS cell line conditionally expressing the dominant active Rab5 mutant, Rab5Q79L was treated for 24h with doxycycline (Q79L+) or left untreated (Q79L-) and concomitantly synchronized at mitosis by addiction of 50 ng/ml of nocodazole for 12 h. Mitotic cells blocked at prometaphase were recovered by mitotic shake off and replated on poly-D-lysine-coated coverslips after nocodazole wash out (NOC release) or in presence of nocodazole (NOC). Cells were fixed 40 min after replating and stained with anti-centrin antibody and DAPI. Left: bar graph showing the % of cells with split centrioles. Mean values ± SD (n=3, 200 cells/condition/experiment) are shown. Rab5Q79L induces centrioles splitting only after nocodazole removal indicating that intact microtubules are required. Right: representative immunofluorescence pictures.Red: centrin,; blue: DAPI..

## Rab5 depletion inhibits centrosomes separation at G2

To establish whether Rab5 participates in centrosome cohesion under physiological conditions, we tested the effect of Rab5-silencing. Based on our findings that all three Rab5 proteins induced centrosome splitting, and that silencing of a single Rab5 protein is not sufficient to reduce endocytosis (Huang et al., 2004), we simultaneously silenced all Rab5 isoforms (Rab5-KD). We performed Rab5 knock-down in U2OS cells stably transfected with the inducible Rab5A-silencing resistant plasmid. Targeting of Rab5 by small-interfering RNAs resulted in ~ 90% depletion of Rab5 proteins. Rab5 ablation was sufficient to cause, as previously reported (Huang et al., 2004), a strong reduction of endocytosis of the transferrin receptor. (Supplemental Fig. 2A)

By treating the Rab5-KD cells with doxycycline, we recovered Rab5A expression to levels comparable to endogenous Rab5A. **(Figure 17)**

In Rab5-KD cells we observed a higher number of H3 (S10-P)-positive cells displaying paired centrosomes compared to the control, indicating that in these cells centrosome separation was inhibited. This was not due to off-target effects since inhibition of centrosome separation was almost completely rescued by re-expression of Rab5A. Moreover, it was not the result of gross alterations in endocytosis as the Eps15Δ95-295 mutant, which blocks clathrin-mediated endocytosis (Benmerah et al., 1999), did not impair centrosome migration at G2. (Supplemental Fig. 2B)

Silencing of Rab5A alone (Rab5A-KD) did not inhibit either centrosome separation, or transferrin endocytosis [(Huang et al., 2004) and data not shown]. Nevertheless re-expression of Rab5A in the cells silenced for all the three Rab5 isoforms was sufficient to rescue centrosome separation. This suggests that, as already shown for the endocytic function of Rab5, the three Rab5 proteins play redundant role also in centrosome separation.

The sum of all these data strongly suggests a participation of Rab5 in centrosome separation at G2 under physiological conditions.

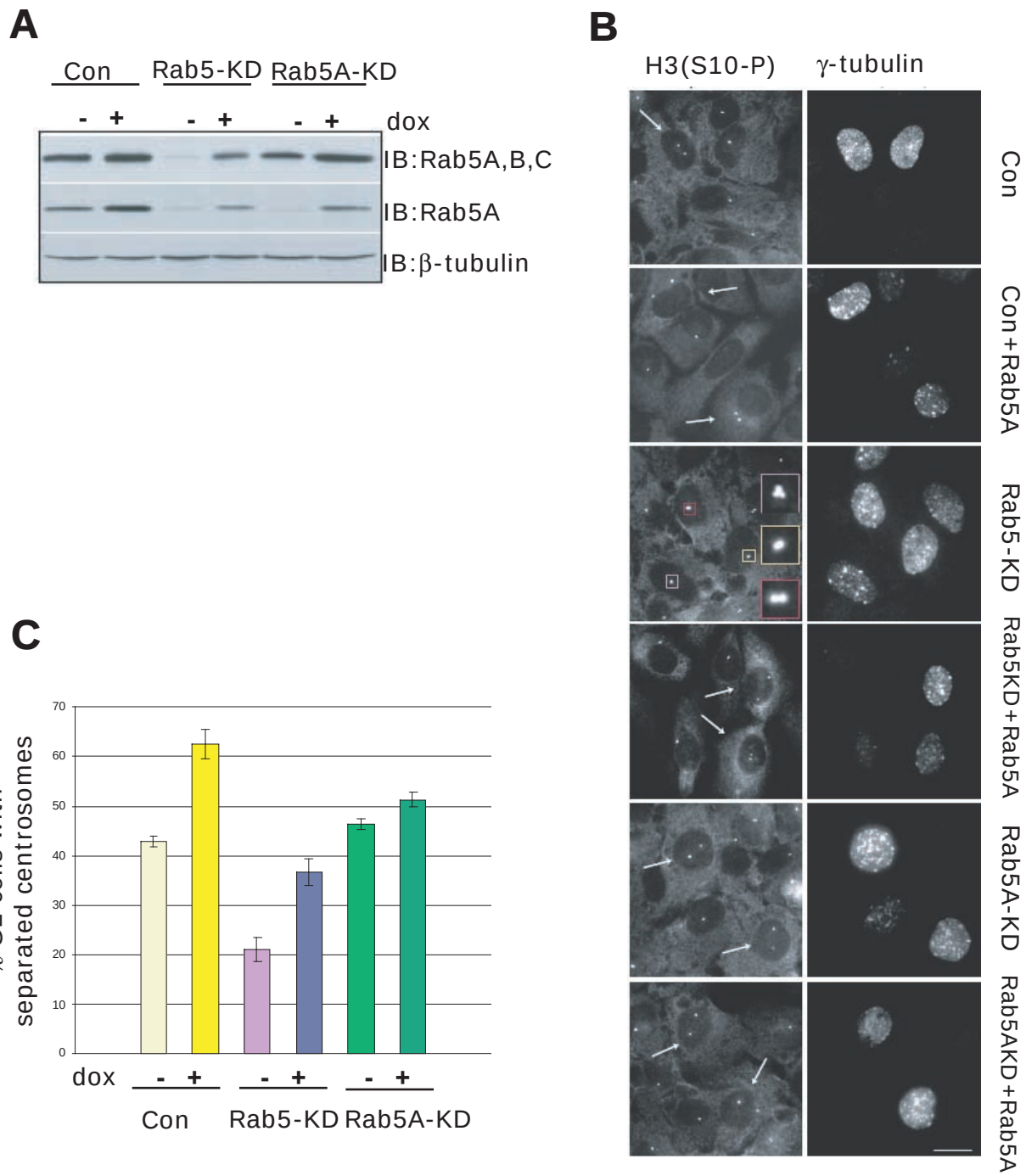

A
Con
Rab5-KD
Rab5A-KD
- + - + - +
dox
IB:Rab5A,B,C
IB:Rab5A
IB:β-tubulin
B
H3(S10-P)
γ-tubulin
Con
Con+Rab5A
Rab5-KD
Rab5KD+Rab5A
Rab5A-KD
Rab5AKD+Rab5A
C
% G2 cells with separated centrosomes
0
10
20
30
40
50
60
70
dox
- + - + - +
Con
Rab5-KD
Rab5A-KD

Figure 17

**Figure 17.** ***Rab5 silencing inhibits centrosome separation during G2.*** U2OS cells stably transfected with the inducible (TET ON) Rab5A-silencing resistant plasmid were transiently transfected with control siRNA oligos (Con.), or with oligos for Rab5A, Rab5B and Rab5C (Rab5-KD) or with an oligo for Rab5A alone (Rab5A-KD). Cell were treated with doxycycline (+) in order to express Rab5A or left untreated (-).

A) Total cellular lysates (50
µg) were IB as indicated.

B) Representative immunofluorescence pictures of the cells treated as in A and indicated on the left, stained with anti-phospho-histone H3 [H3(S10-P)] and anti- γ-tubulin antibodies. Arrows point to G2 cells with separated centrosomes. Paired centrosomes in G2 cells in Rab5-KD are boxed in and magnified in the insets. Conditional reexpression of Rab5A in the Rab5-KD cells is sufficient to recover centrosome separation. Conversely, silencing of Rab5A alone does not affect centrosome separation.

C) Quantification of the experiment shown in B. Mean values ± SD (n=3, 200 cells/condition/experiment) are shown (p value <0.01).

## Evidence for the participation of Rab5 and RN-tre to the same pathway in centrosome cohesion

Recently, a novel GAP for Rab5, RabGAP-5, has been identified (Haas et al., 2005). Moreover it has been shown that RN-tre has GAP activity “in vitro” also on two other GTPases of the Rab family, Rab43 and Rab30 (Haas et al., 2005). Therefore, it was important to provide evidence that Rab5 and RN-tre belong to the same pathway regulating centrosome cohesion.

When RN-tre was co-expressed with Rab5, it was able to counteract the centrosome splitting activity of Rab5. Conversely, co-expression of RabGAP-5 and Rab5 did not result in any attenuation of the Rab5 dependent centrosomal phenotype. It is of note that we could localise RabGAP-5 at the centrosome suggesting that, while probably not directly involved in the Rab5 mediated regulation of centrosome cohesion, this GAP might have a function at the centrosome, possibly connected with Rab5 not evidenced in this study. (Supplemental Fig. 3)

We also tested the ability of Rab43 or Rab30, or their dominant active counterparts (Rab43Q77L and Rab30Q68L) to induce centrosome splitting. None of these proteins displayed significant activity in this assay, despite expression to comparable levels. (Data not shown)

Together our results strongly suggest participation of RN-tre and Rab5 in the same pathway that regulates centrosome cohesion.

## *KIF3A as a possible effector for Rab5 in centrosome regulation*

To gain insight into the mechanism of action of Rab5 in centrosome cohesion, we turned to RN-tre, since it has been previously shown that this protein is endowed with a dual function as Rab5-GAP and Rab5 effector (Lanzetti et al., 2004; Lanzetti et al., 2000). Using the C-terminal region of RN-tre as bait in a two hybrid screen we identified the Kinesin-2 motor protein KIF3A as a novel interactor for RN-tre.

It has been recently published that the Kinesin-2 complex is localised at the centrosomes in interphase and at the spindle microtubule during metaphase (Haraguchi et al., 2005). Moreover Haraguchi et al. found that the expression of a mutant KIF3B, which is able to associate with KIF3A but not with KAP3, caused abnormal spindle formation.

Prompted by this interesting background we investigated the possible role of KIF3A in the Rab5/RN-tre pathway regulating centrosome cohesion. Thereby, we found that KIF3A silencing could prevent the centrosome splitting caused by ablation of RN-tre. In addiction we noticed that KIF3A depletion phenocopies Rab5 silencing and was necessary for RabQ79L induction of centrosome separation at G2.

## RN-tre interacts with KIF3A which is involved in centrosome separation at G2

As the C-terminal region of RN-tre has been found to establish protein-protein interactions and to target the RN-tre at the centrosome, we asked whether it could interact with centrosomal component(s) required for centrosome cohesion. Therefore, we performed a two-hybrid screen using the RN-tre C-terminus, as bait and a human placenta library of preys.

Among the putative interactors, we isolated already known RN-tre-binding proteins (Eps8 and ACTN4) and in addition an interesting novel potential partner: the kinesin motor protein KIF3A. We were able to detect KIF3A in

the anti-RN-tre immunoprecipitates from HeLa cells suggesting that endogenous RN-tre and KIF3A interact *in vivo*. Moreover, *in vitro* pull down experiments confirmed that the interaction between recombinant KIF3A and the RN-tre fragment encompassing positions 447-828 is direct. **(Figure 18A and B)**

To better characterise the meaning of RN-tre and KIF3A interactions we generated an U2OS-derived cell line that conditionally expressed a silencing-resistant variant of KIF3A. Thus, we silenced either KIF3A (KIF3A-KD), RN-tre (RN-tre-KD) or both KIF3A and RN-tre (RN-tre-KD+KIF3A-KD) in these cells. RN-tre depletion caused centrosome splitting which was blocked by the concomitant silencing of KIF3A. Restoration of KIF3A protein expression recovered centrosome splitting in the RN-tre-silenced cells. **(Figure 18C and D)**

These data indicate that the physical interaction between KIF3A and RN-tre uncovers a functional role in the pathway regulating centrosome cohesion.

More importantly, we noticed that ablation of KIF3A inhibited centrosome separation as indicated by a higher number of H3(S10-P)-positive cells with paired centrosomes, the same effect elicited by Rab5 depletion. Centrosome separation was restored in KIF3A-KD cells upon re-expression of KIF3A. **(Figure 18E and F)**

These data unveil a novel function for KIF3A in centrosome cohesion and further implicate this kinesin as a Rab5-downstream effector in centrosome separation.

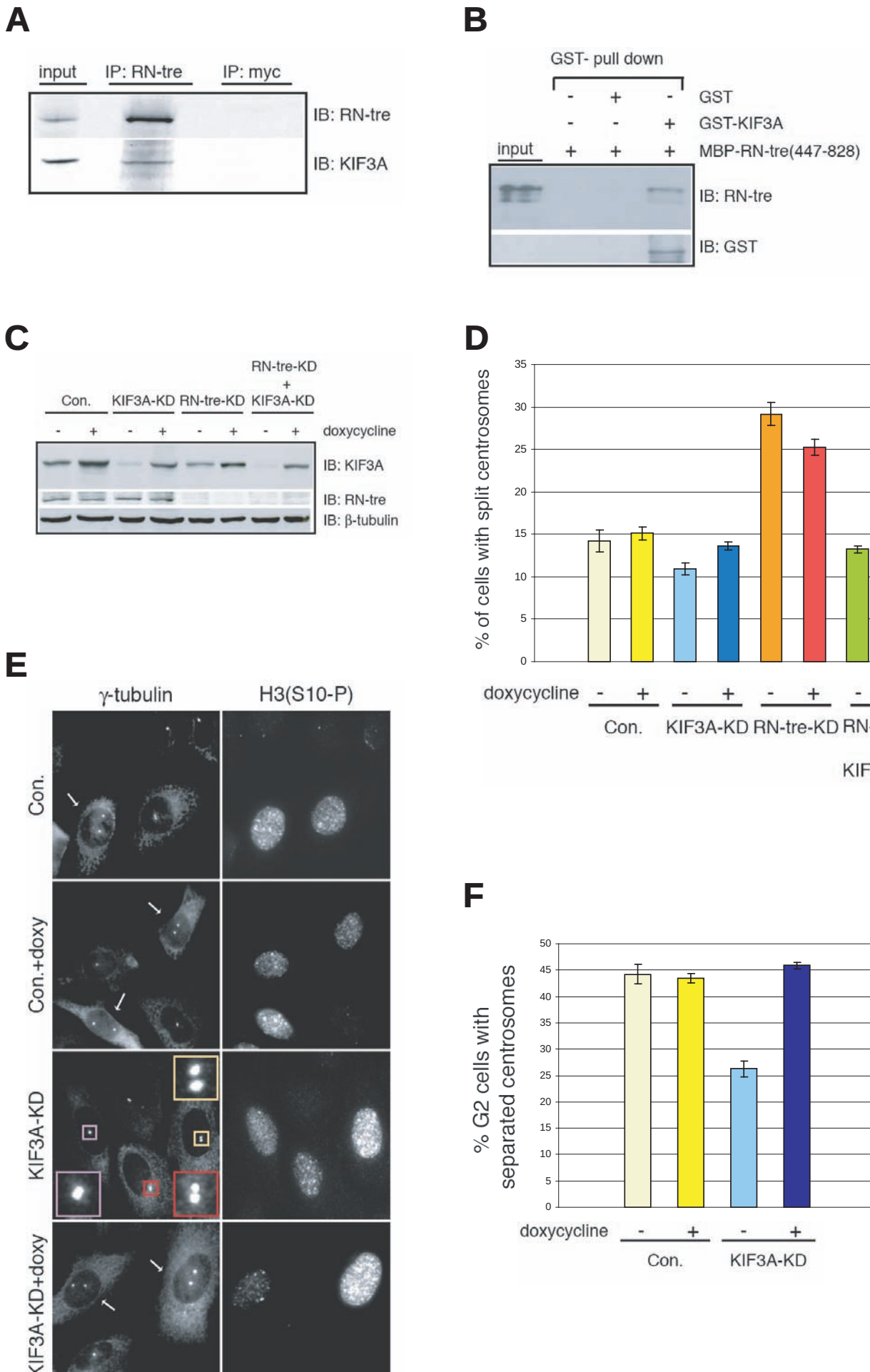

A
input
IP: RN-tre
IP: myc
IB: RN-tre
IB: KIF3A
B
GST- pull down
GST
GST-KIF3A
input
MBP-RN-tre(447-828)
IB: RN-tre
IB: GST
C
Con.
KIF3A-KD
RN-tre-KD
RN-tre-KD + KIF3A-KD
doxycycline
IB: KIF3A
IB: RN-tre
IB: β-tubulin
D
% of cells with split centrosomes
doxycycline
Con.
KIF3A-KD
RN-tre-KD
RN-tre-KD + KIF3A-KD
E
γ-tubulin
H3(S10-P)
Con.
Con.+doxy
KIF3A-KD
KIF3A-KD+doxy
F
% G2 cells with separated centrosomes
doxycycline
Con.
KIF3A-KD

Figure 18

**Figure 18.** ***Requirement for KIF3A, a novel RN-tre-binding partner, in centrosome separation.***

A) Co-immunoprecipitation between endogenous RN-tre and KIF3A. IP: myc, negative control; input, 100 µg of total lysate.

B) GST-pull down between GST-KIF3A and the C-terminal fragment of RN-tre encompassing positions 447-828 fused to MBP [MBP-RN-tre(447-828)]. Equal amounts of GST-KIF3A or GST were bound to glutathione sepharose beads and incubated with purified MBP-RN-tre(447-828) (2 µg). Retained protein was blotted (IB) with mouse monoclonal anti-RN-tre or mouse monoclonal anti-GST antibodies.

C) U2OS cells stably transfected with the inducible (TET ON) KIF3Asilencing resistant plasmid were transfected with the RNAi oligos for control (Con), KIF3A (KIF3A-KD), RN-tre (RN-tre-KD) or RN-tre and KIF3A (RN-tre-KD+KIF3A-KD). Cells were treated with doxycycline (+) to express KIF3A or left untreated. Total cellular lysates of cells treated as in C (80 µg) were IB as indicated.

D) The bar graph shows the percentage of cells (C) with split centrosomes. Mean values ± SD (n=4, 200 cells/condition/experiment) are shown (p value<0.01).

E) U2OS cells stably transfected with the inducible (TET ON) KIF3A silencing resistant plasmid were transfected with the RNAi o ligos for control (Con) or KIF3A (KIF3A-KD), treated with doxycycline (+doxy) to express KIF3A or left untreated and stained with H3(S10-P) and anti-γ-tubulin antibodies. Arrows point at G2 cells with separated centrosomes. Paired centrosomes in G2 cells in KIF3A-KD are magnified and shown in the insets. Conditional re-expression of KIF3A in the KIF3A-KD cells recovered centrosome separation.

G) Quantification of the experiment shown in E. Mean values ± SD (n=3, 200 cells/condition/experiment) are shown (p value<0.01).

## Rab5 requires KIF3A to promote centrosome separation

To test whether KIF3A may act downstream Rab5, we analysed if KIF3A depletion would prevent the increase in centrosome separation observed after Rab5Q79L expression. We silenced KIF3A in the Rab5Q79L inducible cell line described before, and subsequently, we induced expression of Rab5Q79L with doxycycline.

Under these conditions, we achieved ~90% depletion of KIF3A, without any major effect on Rab5Q79L protein levels (compare KIF3A-KD to control). The increase in the number of G2 cells [identified as H3(S10-P)-positive cells] with separated centrosome, caused by the expression of the dominant-active Rab5 mutant, was strongly reduced by KIF3A silencing. **(Figure 19)**

Thus, KIF3A is required for Rab5-induced centrosome separation.

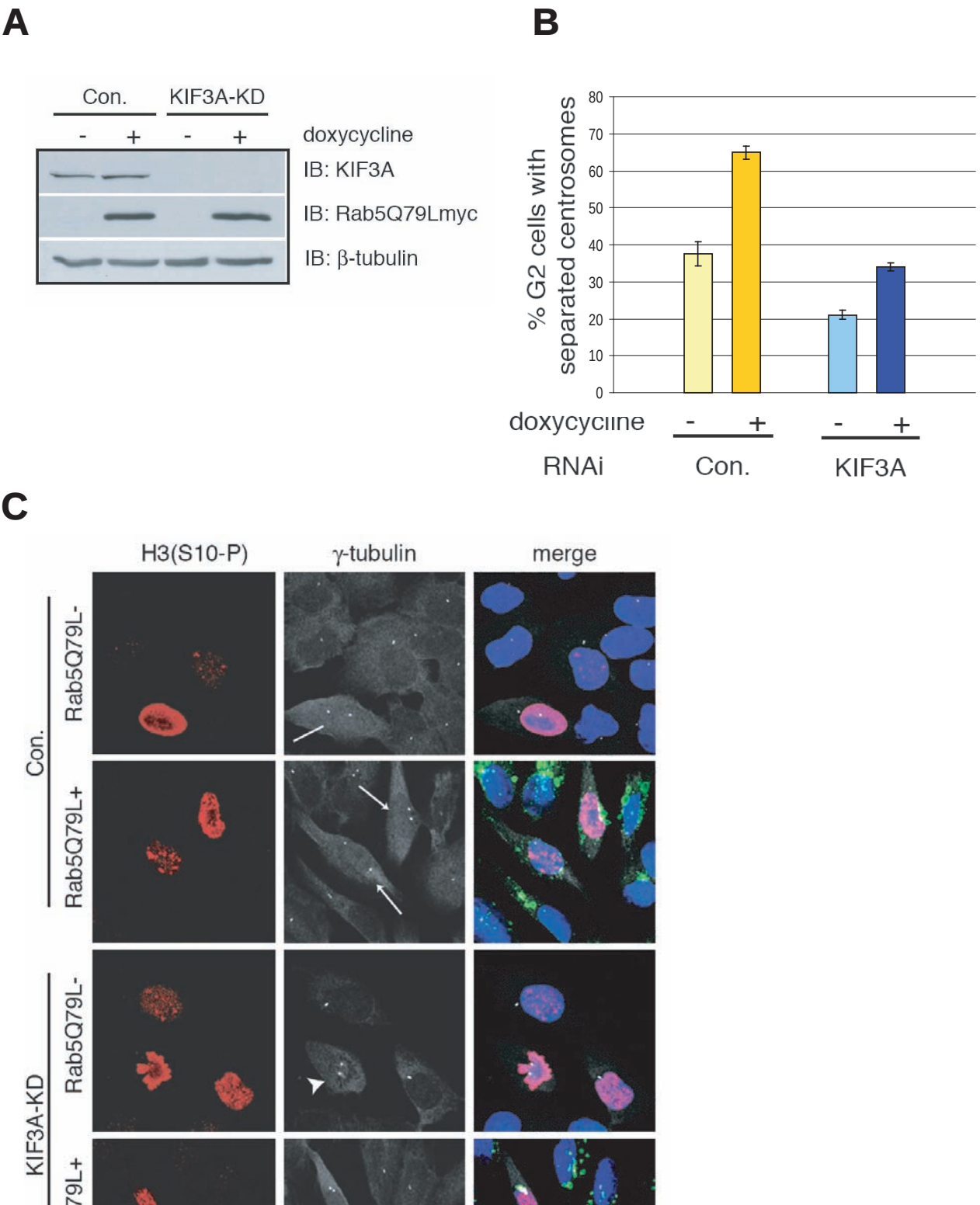

A
Con.
KIF3A-KD
- + - +
doxycycline
IB: KIF3A
IB: Rab5Q79Lmyc
IB: β-tubulin
B
% G2 cells with separated centrosomes
80
70
60
50
40
30
20
10
0
doxycycline - + - +
RNAi Con. KIF3A
C
H3(S10-P)
γ-tubulin
merge
Con.
KIF3A-KD
Rab5Q79L-
Rab5Q79L+
Rab5Q79L-
Rab5Q79L+

Figure 19

**Figure 19.** ***KIF3A is required for Rab5Q79L-induced centrosome splitting***

The Rab5Q79L inducible U2OS cell line was transiently transfected with siRNA oligos for KIF3A (KIF3A-KD) or with control scrambled oligos (Con.). 72 hours later the cells were treated with doxycycline (+) or mock treated (-) for additional 24 h.

A) Total cellular lysates (50 µg) were IB as indicated.

B) Bar graph showing the percentage of H3(S10-P) positive cells with separated centrosomes in the Rab5Q79L clone not-induced (Q79L-) or induced with doxycycline (Q79L+), with or without KIF3A-KD. Mean values ± SD (n=3, 200 cells/condition/experiment) are shown (p value < 0.01).

C) Representative confocal pictures of cells stained with anti-H3(S10-P) (red), anti-γ-tubulin (Cy5, pseudocolored in black and white) anti-myc (to detect Rab5Q79L, green) antibodies and DAPI (blue). The anti-myc and DAPI stainings are shown only in the merge. Arrows point to cells with separated centrosomes. Arrowhead points to a defective mitotic cell in the sample not expressing Rab5Q79L and silenced for KIF3A (Rab5Q79L-).

## *Rab5 and KIF3A affect the formation of the mitotic spindle*

We previously showed that both Rab5 and KIF3A silencing led to the accumulation of G2 cells with unseparated centrosomes without affect the FACS profile of the population. Thus, we asked whether the prevention of centrosome separation has significance in the cell cycle progression.

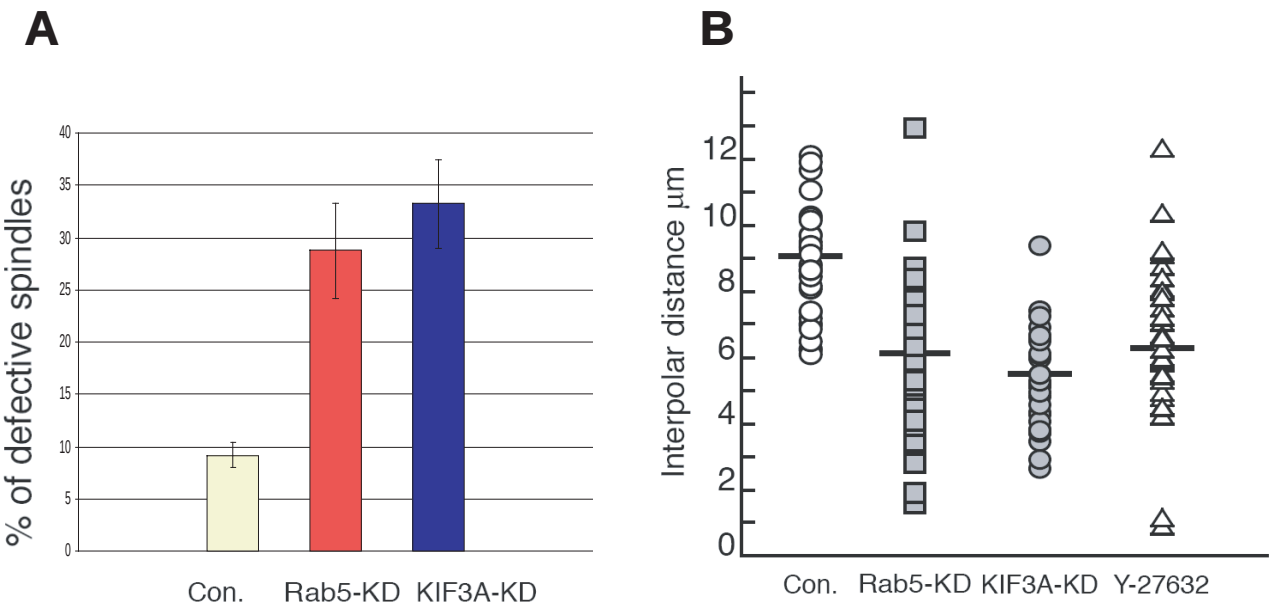
We observed that, as well the FACS profile, the mitotic index was not seriously affected in these samples (data not shown), but we noticed that several Rab5-KD or KIF3A-KD mitotic cells showed defective spindles. In particular most of them appeared to be “shorter” compared to control.

We quantified this effect by randomly acquiring confocal images of mitotic cells and measuring the distance between the spindle poles. Since the Rho kinase inhibitor Y-27632 has been reported to interfere with centrosome separation in PtK2 and B6-8 cells, we used it as positive control. Indeed the interpolar distance in Rab5-KD mitotic cells was reduced compared to the control and this effect was even more pronounced in KIF3A-KD.

We saw that, among the defective spindles, 30%, both in Rab5-KD and KIF3A-KD, showed a “strong” phenotype with barely separated centrosome. Most of the aberrant spindles were characterized by an incomplete alignment of the chromosomes on the metaphase plate (the chromosomes remained mostly at the poles), and a reduced distance between the spindle poles. **(Figure 20)**

Rab5 or KIF3A ablation did not result in a significant percentage of mitotic cells with monopolar spindle, a phenotype caused by inhibition of the mitotic kinesin Eg5 (Blangy et al., 1995), thus, reduction in Rab5 and KIF3A activity strongly affects, but does not completely prevent, migration of the spindle poles.

Further work is needed to better clarify the mitotic phenotype and the execution of cytokinesis in these cells; nevertheless this analysis indicates that both Rab5 and KIF3A depletion result in poor separation of the spindle poles at mitosis.


A
% of defective spindles
40
35
30
25
20
15
10
5
0
Con.
Rab5-KD
KIF3A-KD
B
Interpolar distance µm
12
10
8
6
4
2
0
Con.
Rab5-KD
KIF3A-KD
Y-27632

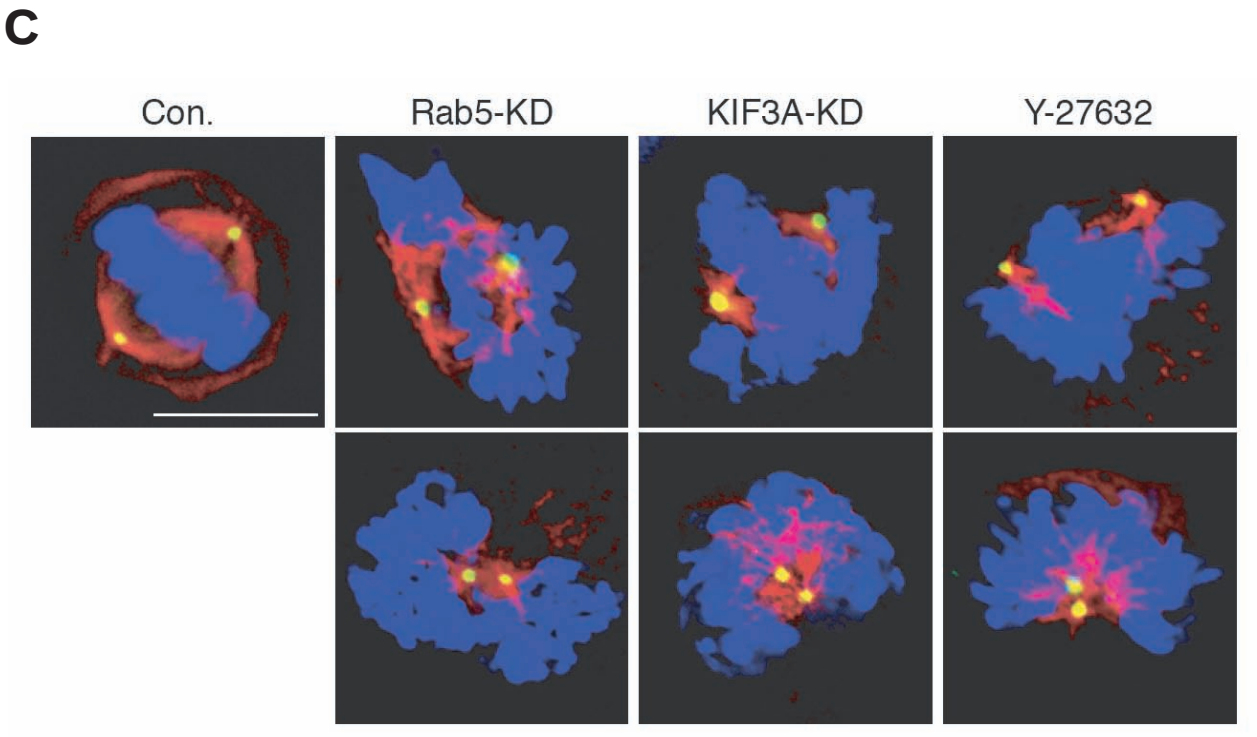

C
Con.
Rab5-KD
KIF3A-KD
Y-27632

Figure 20

**Figure 20.** ***Rab5 or KIF3A depletion results in poor separation of spindle poles at mitosis.***

U2OS cells were silenced for control (Con.), Rab5A, B and C (Rab5-KD) and KIF3A (KIF3A-KD) or treated with Y-27632 (Sigma) for 20 min and stained with anti-γ-tubulin and anti-γ-tubulin antibodies and DAPI.

A) Bar graph showing the % of defective spindles. Mean values ± SD (n=3, 100 cells/condition/experiment) are shown (p value < 0.01).

B) Confocal pictures of mitotic cells from each sample were randomly acquired and the distance between the spindle poles was measured by means of the Profile Quantification Tool of the Leica Confocal Software. The dot plot shows the interpolar distance of 30 mitotic cells randomly and blindly picked from three independent experiments.

C) Examples of mitotic figures. Red: α-tubulin; green: γ -tubulin; blue: DAPI.

# Discussion

In this study we unveil a novel function for Rab5 in the control of centrosome cohesion. This function adds to the previously characterised roles of Rab 5 in endosomal dynamics, early steps of endocytosis, and actin remodelling (Zerial and McBride, 2001).
Herein we have described the localisation of Rab5 and its regulators, RN-tre and Rabex-5, at the centrosome. Performing gain of function and loss of function experiments we found that Rab5 is required for the correct separation of the centrosome during G2 and that reduction in its activity results in the formation of shorter bipolar spindle at mitosis that are likely due to an incomplete separation of the spindle poles.

## *Regulation of Rab5 at the centrosome*

The molecular and biochemical evidence reported here strongly suggest a tight regulation of the centrosomal function of Rab5. Initially we noticed a cell cycle dependent localisation of the small GTPase at that site of action, and, moreover, we found an additional level of fine tuning for Rab5 at that location exerted by positive and negative regulators, Rabex-5 and RN-tre, respectively.
In the simplest scenario the activation of Rab5, promoted by Rabex-5, would be required to induce centrosome separation at prophase, while the activity of RN-tre would prevent unscheduled splitting, by down regulating the GTPase during interphase.
Such a model would, in turn, require additional regulatory mechanisms to control the activity of Rabex-5 and RN-tre.
We noted that the activity of RN-tre can be modulated upon growth factor treatment (Lanzetti et al., 2000), and that RN-tre is also subjected to cell cycle dependent phosphorylation that modulates its GAP activity on Rab5 (Lanzetti et al., 2007).

On the other hand Rabex-5 can be monoubiquitinated in response to stimuli (Penengo et al., 2006). Therefore, it seems that modulation of RN-tre and Rabex-5 could be achieved through post-translational modifications.
Notably, other Rab5 regulators, the GEF Alsin (Millecamps et al., 2005) and the GAP RabGAP-5 (this study) localise to the centrosome pointing out the existence of different regulatory elements for Rab5 at the centrosome.

## *The Rab5-based pathway regulating centrosome cohesion requires the KinesinII motor protein KIF3A*

How does Rab5 induce centrosome separation? Our results implicate both RN-tre and the kinesin motor KIF3A.
Functional ablation experiments indicated that KIF3A is necessary for the centrosome splitting caused by depletion of RN-tre or by dominant-active Rab5. This either implies a function of KIF3A downstream of Rab5 (in the same pathway) or in a parallel (and concomitantly necessary) pathway. The fact that KIF3A physically interacts with the Rab5 regulator RN-tre, supports the first hypothesis.
Interestingly, KIF3A contributes to the efficient separation of centrosome and its ablation results in defective spindles similar to those caused by Rab5 silencing strengthening the notion that Rab5 and KIF3A are part of a novel mechanism that participates to the formation of a normal bipolar spindle.
KIF3A is part of kinesin II, a microtubule plus end-directed motor complex that transports both vesicles, like the recycling endosomes (Imamura et al., 2003) and the late endosomes (Brown et al., 2005), and macromolecular complexes such as the flagellar components in ciliated cells (Rosenbaum et al., 1999) or soluble enzymes along the axons (Ray et al., 1999). Our results now also implicate kinesin II in centrosome separation.
Our functional evidence is corroborated by previous findings showing that i) a fraction of KIF3A is associated to the centrioles (Haraguchi et al., 2006; Vashishtha et al., 1996); ii) mutations that disrupt the binding of KIF3A or KIF3B to KAP3 result in the assembly of multiple spindles at mitosis in NIH cells (Haraguchi et al., 2006); a phenotype similar to that caused by

expression of the dominant-active Rab5 mutant, or by silencing RN-tre; iii) point mutants of both the two motor subunits show an increased frequency of chromosomes loss in Chlamydomonas (Miller et al., 2005). Collectively these data support our findings that KIF3A participates in centrosome cohesion.

While we did not investigate the molecular mechanisms that cause centrosome splitting upon overexpression of Rab5 and Rabex-5 and that might also have different underpinnings, we have revealed a novel function for Rab5 and KIF3A in centrosome separation at physiological levels.

Importantly, it was recently demonstrated that Rab5 controls, during interphase, the activity of another kinesin, KIF16B, involved in the transport of early endosomes to the plus end of microtubules (Hoepfner et al., 2005). In turn, KIF16B is required for the steady-state distribution of early endosomes and for their correct recycling function (Hoepfner et al., 2005). Extensive studies on the modality of action of Rab5 have clearly shown that active Rab5 functions by creating signalling platforms and recruiting downstream effectors (Lippe et al., 2001; Zerial and McBride, 2001). From the combined analysis of our data and those of Hoepfner et al. (Hoepfner et al., 2005), it appears that control over motor proteins is a relevant function of Rab5.

## *Biological implication for the control of centrosome cohesion by Rab5*

One attractive idea is that Rab5 determines the timing of parental centrioles disjunction by activating or recruiting downstream effectors at the centrosome. The localisation of Rab5 close to the centrosome from interphase to prophase-prometaphase and its release at metaphase, when separation of spindle poles has brought to completion, nicely fits with this possibility. Notably KAP3, the cargo-binding subunit of kinesin II, which couples the motor to its targets, is only targeted to the centrosome between prophase and prometaphase (Haraguchi et al., 2006), compatible with the need for Rab5 effector function at the G2/M interface.

It is tempting to speculate that Rab5 might control a non-essential checkpoint that couples centrosome separation with yet unknown functions to be executed before mitosis.

During mitosis, the biosynthetic activity in animal cells slows down, and the main task is then to segregate the chromosomes and to all other cellular components to the two daughter cells. Given the pivotal role of Rab5 in endosomal dynamics, ordered endosomal inheritance, a process in which microtubules are thought to have an important role (Bergeland et al., 2001; Dunster et al., 2002), might represent a candidate for this hypothetical function.

# Future perspectives

## *Studying the interaction between Rab5 and KIF3A in vivo*

The sum of our experiments strongly suggests that KIF3A acts downstream of Rab5 in the regulation of centrosome cohesion. Moreover, KIF3A binds directly to the Rab5 regulators RN-tre.

Thus, in the Rab5-based pathway regulating centrosomes cohesion RN-tre could exert the dual function of Rab5-GAP and Rab5 effector connecting Rab5 signals to the microtubules cytoskeleton. Indeed, a more complex scenario is emerging.

Our preliminary data, in fact, indicate a direct interaction between Rab5 and KIF3A *in vitro*. In pull down assay experiments, performed using the purified proteins, KIF3A co-precipitates with GST-Rab5. Furthermore, the affinity of KIF3A for Rab5 increases when the GTPase is loaded with GTPγS instead of GDP.

It has been previously shown that activated Rab4, but not Rab5, can bind to the kinesin family protein KIF3 (Imamura et al., 2003). In the Imamura et al study, the interaction with Rab4, Rab5 and KIF3 was analysed by co-immunoprecipitation or by pull down of the total cell lysate with the purified GST-Rab4 and -Rab5. These findings appear to conflict with our results.

Since the centrosome separation takes place at a specific time point of the cell cycle one can speculate that the *in vivo* interaction between Rab5 and KIF3A is restricted to that moment. To further investigate this hypothesis pull down experiments on lysates obtained from cells synchronised in different phases of the cell cycle will be performed.

## *Live cell imaging approaches to further investigate the role of Rab5 at the centrosome*

New and updated fluorescence imaging methods allowed us to determine the subcellular localisation, motility and transport pathways of specific protein and even to visualise protein-protein interactions of single molecules in living cells. Direct observation of such molecular dynamics can provide important information about cellular events that cannot be obtained by other methods.

We described that Rab5 localises at the centrosome in a cell cycle dependent manner. To further investigate the relationship between Rab5 and the centrosome it would be interesting to perform FRAP experiment and analyse the fluorescence recovery related to different centrosome cycle phase.

The analysis performed by fluoresce imaging on fixed cells only provides a static, snapshot view of the Rab5 behaviour. Being able to observe processes as they happen within the cell by light microscopy would increase our understanding about the relationship between endocytosis and cell division.

We plan to follow the endocytic process in living cells overexpressing Rab5 in order to evaluate the possible existence of two different Rab5 sub-populations: one localising at the centrosome and the other one on the early endosomes.

To this purpose we will use stable cell line expressing GFP-Centrin, in order to visualize the centrosome during time. The endocytic process will be follow in cells transiently transfected with RFP-Rab5 and treated with fluorescent dextran conjugates. Fluorescent dextran conjugates have been used to monitor the uptake and internal processing of exogenous materials by endocytosis and may also be useful for studies of endosome fusion, cell membrane changes, and vesicular morphology (Plank et al., 1994; Devon et al., 2006; Mrotta et al., 2006 ).

Our final goal is to demonstrate the existance of a dynamic relationship between plasma membrane modifications and centrosome cohesion and,

possibly, a direct involvement of endocytosis in the regulation of cell cycle progression.

We showed that the ectopic expression of Rab5Q79L as well as RN-tre silencing induced mitotic aberration leading to the formation of multipolar spindles. On the other hand we observed that Rab5 and KIF3A silencing resulted in the formation of shorter spindle characterised by an incomplete alignment of the chromosomes on the metaphase plate. Prompted by these findings we would like to perform a detailed analysis of the mitotic progression after the disruption of the physiological level of Rab5, KIF3A or RN-tre.

We could not expect a gross alteration in the duration of the division process since the mitotic index in such populations seems to be unmodified compared with the control. However, we did expect to find alterations in the accuracy of chromosomes segregation and in the completion of cytokinesis since the centrosomes directly activate cleavage furrow (Piel et al., 2001).

Of note, RabQ79L overexpressing cells and RN-tre KD cells, as well as Rab5-KD cells, analysed after 48 hours of culture, showed unaffected FACS profiles. These results could be due to the fact that more replication cycles are needed to underline DNA quantities abnormalities in these cells.

In order to analyse the cell division process we generated a stable cell line expressing GFP-α-tubulin. The presence of the GFP-α-tubulin added to the treatment with lipophilic Hoechst, a fluorescent stain that binds to DNA, allows us to follow the spindle formation and the cell cycle progression during *in vivo* time-lapse experiments.

These kinds of experiments will contribute relevant insights into the Rab5-based pathway in centrosome cohesion and in the outcome of cell division.

## *Studying the relevance of RN-tre/KIF3A interaction in different cellular process*

We demonstrated that RN-tre binds to KIF3A both *in vitro* and *in vivo*. Moreover, the physical interaction between these two proteins uncovers a functional role in the pathway regulating centrosome cohesion though, we cannot exclude that this interaction could exert a relevant function also in other cellular processes.

In a recent study RN-tre has been shown to have a role in the biogenesis and maintenance of functional Golgi structure: the overexpression of RN-tre causes activity dependent fragmentation of both the cis-Golgi marker GM130 and the trans-Golgi marker TGN46 (Haas et al., 2007). Moreover, RN-tre is required for retrograde trafficking to trans-Golgi, but not anterograde cargo transport through the Golgi (Fuchs et al., 2007).

Rab5 does not seem to be involved in this process, however, other Rab GTPases could partake in this transport since it has been demonstrated that RN-tre displayed a promiscuous GAP activity in vitro (Hass et al., 2005).

At the end of 2006 Stauber and co-workers demonstrated that KAP3 silencing as well as KIF3a silencing results in the fragmentation of the Golgi apparatus and they suggested a role for kinesin-2 in the retrograde transport pathway from the Golgi complex to ER (Stauber et al., 2006).

These recent findings suggest a possible cooperation between RN-tre and KIF3A in the regulation of the retrograde transport from the Golgi apparatus. Thus, it would be interesting to further investigate the meaning of RN-tre/KIF3A interaction at this site.

# Experimental procedures

## *Expression vectors*

The plasmids pEGFP-RN-tre, pCDNAHA1-RN-tre and pEGFP-RN-treR150 were described previously (Lanzetti et al., 2000). YFP-Rab5A, YFP-Rab5B and YFP-Rab5C were kindly provided by A. Sorkin. pEGFPC2-Rabex-5 was a gift from L. Penengo. GFP-Rab5 and pCDNAIIIRab5Q79Lmyc were kindly provided by M. Zerial. The Rab30 cDNA was kindly provided by D. Lambright. The Rab21 constructs were from J. Ivaska while the Rab22 constructs were from V.M. Olkkonen. All other plasmids used in this study were engineered by standard molecular biology techniques and further details are in Engineering of constructs. All constructs were sequence verified.

## *Engineering of constructs and their expression in U2OS cells*

GFP-based constructs were engineered by PCR or by endonuclease digestion and subcloned in the pEGFP-C1 (Clontech) vector. Details are available upon request.

Rab41 and RabGAP-5 were amplified by PCR with specific primers from the I.M.A.G.E.

clones ID 1736002 (Rab41) and ID 3027777 (RabGAP-5) and cloned respectively in the pEGFPC1 and pEGFPC2 vectors (Clontech). Rab30 was amplified by PCR from the GSTRab30 construct provided by D. Lambright and cloned in the pEGFPC1 vector. The mutants Rab41Q90L and Rab30Q68L were generated by recombinant PCR and cloned in pEGFP vectors.

To generate the inducible Rab5Q79L clone, Rab5Q79Lmyc was excised with BamHIXhoI from pCDNAIIIRab5Q79Lmyc and cloned, in the tetracycline-inducible vector pSG213 (a gift from G. Draetta) digested with BglII-XhoI. For the ablation/reconstitution experiments (Fig. 3 and Fig.5 of the main text), the inducible silencing-resistant Rab5A and KIF3A plasmids were generated by recombinant PCR mutating three nucleotides in the sequence targeted by silencing oligos, described in the main text, without affecting the amino acid sequence and cloned in the tetracycline-inducible vector pSG213.

All constructs were sequence verified and further details are available upon request.

For transient transfection of plasmids in U2OS cells, the Fugene-6 transfection reagent

(Roche) was employed according to the manufacturer's instructions.

Derivatives of U2OS conditionally expressing Rab5Q79Lmyc allele or empty vector or

the silencing-resistant version of Rab5A or KIF3A alleles were generated by transfecting the corresponding plasmids with lipofectamine according to the manufacturer's instructions and selecting stably transfectants with puromycin (2 μg/ml, Sigma).

## *Cell culture and transfection*

U2OS cells were grown in DMEM supplemented with 10% fetal bovine serum (Gibco).

U2OS derivative inducible cell lines were grown in DMEM supplemented with 10% Tet

system-approved FBS (Gibco). RNA interference for RN-tre, performed by transient transfection of siRNA oligos, was described previously (Lanzetti et al., 2004). RNA interference for Rab5 was performed using siGENOME SMART pool siRNA oligos for Rab5B and Rab5C, and the following single Rab5A-specific oligo: 5'-AGGAATCAGTGTTGTAGTATT-3' at a final

concentration of 20 120 nM. RNA interference for KIF3A was performed with the oligo 5'- TATCGTAACTCTAAACTGATT-3' at a final concentration of 100 nM. In the RNAi experiments cells were transfected twice with Oligofectamine (according to manifacture instructions) and harvested 96 h after the first transfection. Silencing oligo duplexes were purchased from Dharmacon. Expression of the silencing-resistant constructs Rab5A or KIF3A in the inducible cell lines was achieved by addiction of doxycycline (5 µg/ml, Sigma) 48 h after transfection of siRNA oligos. Controls were performed with siCONTROL Non- Targeting siRNA (Dharmacon).

## *Antibodies*

Antibodies used were: an affinity-purified rabbit polyclonal anti-RN-tre, and a mouse monoclonal anti-RN-tre, described previously (Lanzetti et al., 2004); a mouse monoclonal anti-Rabex-5 (G32), described previously (Penengo et al., 2006); a mouse monoclonal anticentrin 20H5 kindly provided by J.L. Salisbury. All other antibodies were from commercial sources.

Commercial antibodies were: mouse monoclonal anti-γ-tubulin (mouse ascite fluid clone GTU-88, Sigma); rabbit polyclonal anti-γ-tubulin (Sigma) rabbit polyclonal anti-β-tubulin (H-235, Santa Cruz); mouse monoclonal anti-Nek2 (Transduction Laboratories); rabbit polyclonal anti-Rab5 (anti-pan Rab5); mouse monoclonal anti-Transferrin receptor (13-6890 Zymed); rabbit polyclonal anti-Rab5A (s-19, Santa Cruz); mouse monoclonal anti-Rab5 clone1 Becton Dickinson; mouse monoclonal anti-Mcm7 DCS141 ; mouse monoclonal antimyc (9E10, Santa Cruz); rabbit polyclonal anti-phospho histone H3(S10-P) (Cell Signaling); mouse monoclonal anti-α-tubulin (mouse ascites fluid clone B-5-1-2, Sigma); rabbit polyclonal anti-HA (Y-11, Santa Cruz), anti-vinculin (hVIN-1, Sigma); mouse monoclonal anti-KIF3A (BD Transduction Laboratories); mouse monoclonal anti-GST antibody (B-14, Santa Cruz).

## *Microscopy*

For IF experiments, cells were grown on glass coverslips coated with gelatin and

immunostained with the indicated antibodies. Cytosol depletion (when indicated) was

performed by treatment with extraction buffer (20mM Hepes pH 7.5, 50 mM NaCl, 3 mM MgCl2, 300 mM sucrose, 0.5% Triton X-100) for 5 min, prior to methanol fixation. Primary antibodies were revealed by Alexa Fluor 555-, 488- (Molecular Probes) or Cy5- (Jackson) conjugated secondary antibodies. Images were captured using an inverted photomicroscope (DM IRB HC; Leica Microsystems) equipped with mercury short arc epifluorescence lamp, appropriate combination of filters and a cooled digital CCD Hamamatsu ORCA camera (Hamamatsu Photonics), digitally recorded with ImageproPlus 4.0 imaging software (Media Cybernetics) and processed with Adobe Photoshop 8.0. Confocal analysis was performed on a Leica TCS SP2 AOBS microscope.

## *Fluorescence recovery after photobleaching experiments*

Fluorescence recovery after photobleaching (FRAP) was performed on an LSM510 confocal microscope (Zeiss) with a heated stage and a 40x oil-immersion lens.

U2OS cells were plated on Lab-Tek chambered coverglass (Nunc) and transfected with a construct encoding GFP-RN-tre574-828. For live-cell recording, the cells were supplied with a phenol-red free, CO2- independent medium (Invitrogen). For microtubule depolymerization experiments, cells were incubated with 5 µg/ml nocodazole (Sigma) at 37oC for 2-4 h prior to imaging. Staining of included coverslips with anti-α-tubulin antibody confirmed that microtubules had indeed been depolymerized by nocodazole treatment. In all FRAP experiments, a square was centered on one

centrosome and after acquisition of five prebleach images this region was bleached with 3 iterations of 100% laser power at an excitation wavelength of 488. Fluorescence recovery was subsequently followed by acquisition of 100 images, every 96 msec, using an excitation wavelength of 488 nm and 0.5% laser transmission. For each time point, the fluorescence intensity of the photobleached region was determined using LSM510 software. Averaged fluorescence intensities were normalized to the post-bleach value allowing for more accurate kinetic modeling using the equation: Post NFU= (It-Io)/(I-Io), as described previously (Bekker-Jensen et al., 2005). The fluorescence values of individual frames were annotated so that It denotes the intensity of the measured centrosome region at time point t, whereas Io and I denote the values measured in the first and last frame, respectively. Post-normalization of fluorescent units (Post NFU) translates thedata into a kinetic profile of numbers between 0 and 1. Recorded images were subsequently processed with Adobe Photoshop 8.0.

## *Biochemical assays*

For co-immunoprecipitation on endogenous proteins HeLa cells were lysed on ice with

RIPA buffer (10 mM Tris HCl pH 7,6, 150 mM NaCl, 2 mM EDTA 1% Triton X-100, 1%

Na deoxycholate, 0,1% SDS), followed by 1:2 dilution in the same buffer without detergent, 10 mg of lysates were IP using either the monoclonal anti-RN-tre or anti-myc antibodies. Immunoprecipitation on overexpressed proteins was performed with the anti-HA antibody with 1 mg of total cellular lysates from 293T cells transfected with the plasmids indicated in the legend. Immunoblotting was as described (Fazioli et al., 1993).

## *Purification of recombinant proteins and pull down assays.*

To produce recombinant GST-KIF3A and MBP-RN-tre(447-828), pGEX4T.1-KIF3A and

pMALc2X-RN-tre(447-828) were transformed into protease-deficient *E. coli* BL21 and the cells were grown to an OD600 of 0.6 before expression of fusion proteins were induced with 0.1 mM isopropyl-1-thio-b-D-galactopyranoside (IPTG) for 3 hrs at 37oC. The recombinant GST-KIF3A and MBP-RN-tre(447-828) were purified from bacterial lysates on glutathione sepharose beads (Amersham) and on amylose resin (New England Biolabs), respectively, according to the manufacturers instructions. GST-KIF3A and MPB-RN-tre(447-828) were eluted with 20 mM glutathione and 10 mM maltose, respectively, and both proteins were dialyzed prior to use.

For in vitro binding assays, GST and GST-KIF3A affinity matrices were prepared by

adding 10 mg of each protein to 40 ml 50% slurry of glutathione-sepharose beads in 400 ml ELB binding buffer (50 mM Hepes pH 7.0; 250 mM NaCl; 0.1% NP-40 + protease inhibitors). Each mixture was incubated on a rotating wheel for 1 hr at 4oC. The supernatant was removed, and each of the affinity matrices was suspended in 400 ml ELB binding buffer and added 2 mg purified MBP-RN-tre(447-828) protein. After 2 hrs incubation at 4oC on a rotating wheel, the affinity matrices were washed 5 times with 1 ml of ELB binding buffer. Washed resin was suspended in 20 ml 2x SDS PAGE loading buffer, boiled for 5 min and bound protein analyzed by SDS-PAGE on 10% protein gels.

## *Biochemical purification of centrosomal fractions*

For a single centrosome preparation, approximately 2 x 109 KE37 cells in suspension were used. Cells were initially treated with 60 ng/ml nocodazole (Sigma) and 1 µg/ml

Cytochalasin D (Sigma) for 1 h at 37oC. All subsequent steps were performed at 4oC. Cells were rinsed and concentrated in TBS (10 mM Tris-HCl pH 7.4, 150 mM NaCl) followed by a wash in a buffer containing 8% (w/v) sucrose (TBS-sucrose, 1 mM Tris-HCl pH 7.4, 15 mM NaCl, 8% sucrose). Cells were resuspended in TBS-sucrose, and lysis buffer was added (1 mM Hepes pH 7.2, 0.5% NP-40, 0.5 mM MgCl2, 0.1% β-mercaptoethanol, 1 mM PMSF, 1 µg/ml leupeptin and aprotinin) to obtain a final concentration of 1 x 107 cells/ml. Nuclei and other cell debris were subsequently removed by centrifugation, and lysate were filtered through medical gauge before incubation with 10 µg/ml DNase I for 30 min. The cell lysate was then layered onto a 50% sucrose cushion (5 ml 50% sucrose in 10 mM Pipes pH 7.2, 0.1% Triton X-100, 0.1% β-mercaptoethanol) in 38-ml Beckman polyallomer centrifuge tubes, and centrifuged at 11,000 rpm for 20 min in an LKB (Ultrospin 70) ultracentrifuge using a

SW27 swinging bucket rotor. After centrifugation most of the lysate above the gradient interface was discarded. The remaining lysate and gradient was gently mixed and layered onto a discontinuous sucrose gradient in Beckman polyallomer centrifugation tubes. The discontinuous sucrose gradient consisted of 5 ml 70% sucrose, 3 ml 50% sucrose and 3 ml 40% sucrose all dissolved in gradient buffer (10 mM Pipes pH 7.2, 0.1% Triton X-100, 0.1% β-mercaptoethanol). The gradient was centrifuged at 25,000 rpm for 80 min. After centrifugation, 0.5 ml fractions were collected through a hole in the bottom of the tube. Equal volume aliquots of the fractions were analyzed by SDS-PAGE on 10% gels followed by immunoblotting. Fractions 1-16 were tested and peak centrosomes were present in fraction 7- 8. The integrity of purified centrosomes was further validated by immunofluorescence with anti-γ-tubulin and anti-centrin antibodies.

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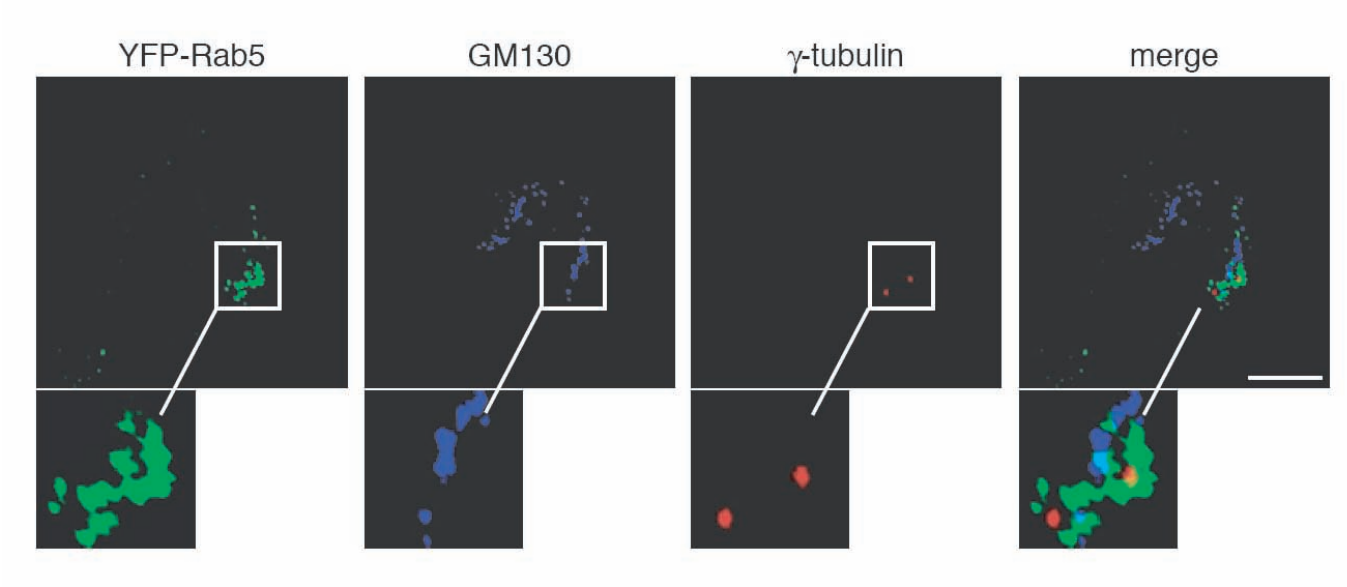
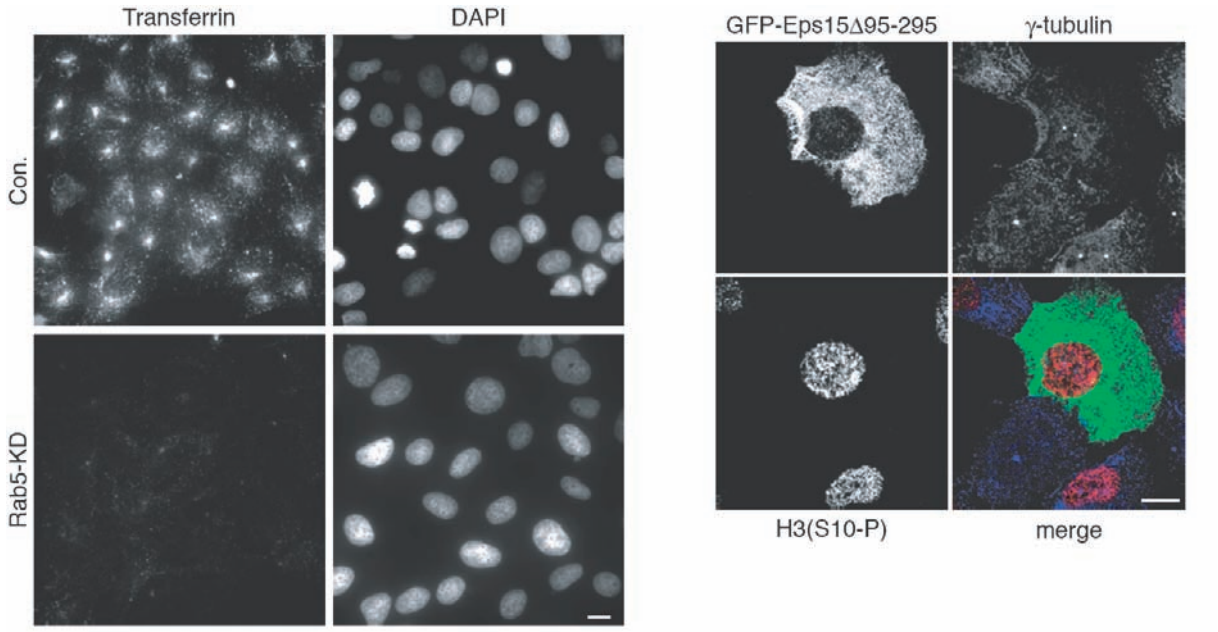
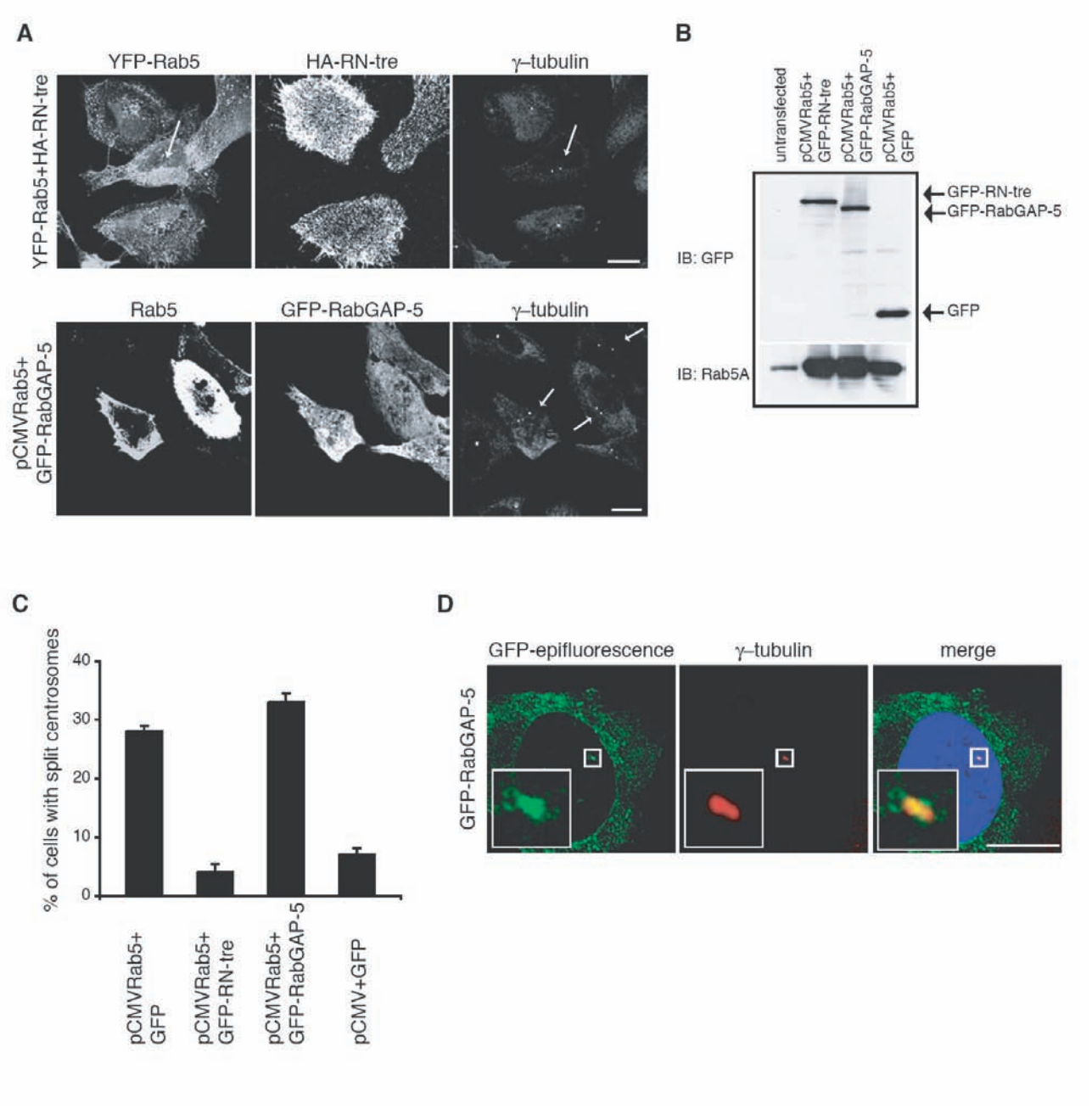



**Supplemental Fig. 1. *YFP-Rab5 does not localize to the Golgi*.**
Confocal picture of U2OS cell transfected with YFP-Rab5 (green), and stained with GM130 (blue) and anti-γ-tubulin (red). Merge is also shown. The centrosomal region is magnified in the insets. Bar, 10 μm.




**Supplemental Fig. 2.** ***Rab5 silencing inhibits transferrin uptake.***
The Eps15Δ95-295 mutant does not prevent centrosome separation at G2. Centrosome duplication is not significantly affected in Rab5-KD cells.
A) U2OS cells, silenced as shown on the left were serum starved for 2 h. Alexa Fluor 555-conjugated transferrin (Invitrogene) was added to the medium at a final concentration of 5 µg/ml, and the cells were allowed to internalize the ligand for 15 min at 37°C, prior to fixation and DAPI staining.
B) U2OS cells were transfected with GFP-Eps15Δ95-295 and stained with anti-H3(S10-P) and anti-γ -tubulin antibodies. Merge shows GFP-Eps15Δ95-295 in green, H3(S10-P) in red, and γ-tubulin in blue. Both the untransfected and the GFP-Eps15Δ95-295 expressing cells are at late G2, and have split centrosomes. In A and B, pictures are representative of three independent experiments, and bars are 10 µm.




**Supplemental Fig. 3.** ***RN-tre and RabGAP-5, in centrosome cohesion.***

A) U2OS cells were transfected with either YFP-Rab5 and HA-RN-tre (top), or pCMVRab5 and GFPRabGAP-5 (bottom), and detected in IF as indicated on top. Top panels, the arrows point to a cell expressing only YFP-Rab5, and displaying split centrosomes, whereas surrounding cells,co-expressing Rab5 and RN-tre, have paired centrosomes. Bottom panels, arrows point to cells expressing both Rab5 and RabGAP-5, and displaying split centrosomes.

B-C) U2OS cells were transfected with the indicated plasmids and assayed in IB as indicated (B, 50 μg total lysate/lane), and by IF to score split centrosomes (C). In C, the bar graph shows mean values ± SD (n=5, 200 cells/condition/experiment).

D) Confocal analysis of U2OS cell transfected with GFP-RabGAP-5 (green) and stained with anti-γ-tubulin (red). Merged images are also shown (blue, DAPI). The centrosomal region is boxed in and magnified in the insets.