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GSK-3β-regulated interaction of BICD with dynein is involved in microtubule anchorage at centrosome

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Microtubule arrays direct intracellular organization and define cellular polarity. Here, we show a novel function of glycogen synthase kinase-3β (GSK-3β) in the organization of microtubule arrays through the interaction with Bicaudal-D (BICD). BICD is known to form a complex with dynein–dynactin and to function in the intracellular vesicle trafficking. Our data revealed that GSK-3β is required for the binding of BICD to dynein but not to dynactin. Knockdown of GSK-3β or BICD reduced centrosomally focused microtubules and induced the mislocalization of centrosomal proteins. The unfocused microtubules in GSK-3β knockdown cells were rescued by the expression of the dynein intermediate chain-BICD fusion protein. Microtubule regrowth assays showed that GSK-3β and BICD are required for the anchoring of microtubules to the centrosome. These results imply that GSK-3β may function in transporting centrosomal proteins to the centrosome by stabilizing the BICD1 and dynein complex, resulting in the regulation of a focused microtubule organization.

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Introduction

Microtubules are essential for various cellular functions, including vesicle transport and cell motility, polarity, and division. The precise microtubule patterns deployed in cells are important for these microtubule-dependent cellular processes. In most vertebrate cells, microtubules radiate from a centrally located centrosomal nucleating center (Doxsey, 2001). Centrosome-dependent organization of microtubules involves distinct processes, such as nucleation, anchoring, and release of microtubules. Microtubules are nucleated by γ-tubulin ring complexes (γ-TuRC) within the pericentriolar materials. γ-Tubulin initiates nucleation by forming rings that act as templates for new microtubule growth (Meads and Schroer, 1995). After nucleation, microtubules grow out with their plus ends leading into the cytoplasm. The minus ends of microtubules are usually anchored in the pericentriolar material (Dammermann *et al*, 2003). Several proteins, including ninein, PCM-1, and centrin 3, are required for the formation and maintenance of a radial microtubule array anchored at the centrosome in interphase (Mogensen *et al*, 2000; Dammermann and Merdes, 2002). Ninein is specifically associated with microtubule minus ends in various cell lines and participates in microtubule anchoring at the centrosome (Mogensen *et al*, 2000). In addition, the dynein–dynactin complex has been shown to be involved in the anchoring of microtubules (Quintyne *et al*, 1999). However, how these proteins are functionally integrated to regulate the microtubule anchoring to the centrosome is unclear.

The serine/threonine kinase glycogen synthase kinase-3 (GSK-3) was first described in a glycogen metabolic pathway (Plyte *et al*, 1992). GSK-3 is highly conserved through evolution and plays a fundamental role in cellular responses. There are two GSK-3 isoforms, GSK-3α and GSK-3β, in mammalian cells and both GSK-3 proteins regulate several physiological responses by phosphorylating many substrates, including protein synthesis, gene expression, subcellular localization of proteins, and protein degradation (Cohen and Frame, 2001; Jope and Johnson, 2004).

Evidence has been accumulated to show that GSK-3 regulates microtubule dynamics (Cohen and Frame, 2001; Jope and Johnson, 2004). Two microtubule-associating proteins (MAPs), Tau and MAP1B, are phosphorylated by GSK-3, and the phosphorylation of MAPs regulates their binding to microtubules, thereby modulating microtubule stability. GSK-3, which is inactivated on the plus ends of microtubules, mediates Par6-protein kinase C ζ-dependent promotion of polarization and cell protrusion through microtubules (Etienne-Manneville and Hall, 2003). The binding of adenomatous polyposis coli (APC) gene product to microtubules increases the stability of microtubules, and the interaction of APC and microtubules is decreased by the phosphorylation of APC by GSK-3β (Zumbrunn *et al*, 2001). However, the roles of GSK-3 at the microtubule minus ends are largely unknown. Here, we show that the interaction of GSK-3β with Bicaudal-D (BICD) is required for the maintenance of microtubule anchoring to the centrosome probably through the centrosomal localization of centrosomal proteins.

Results

Involvement of GSK-3β in the anchoring of microtubules

When cells were stained with anti-GSK-3β antibody (Figure 1A, mouse monoclonal antibody; Supplementary Figure 1A, rabbit polyclonal antibody), large amounts of endogenous GSK-3β were mainly diffusely distributed throughout the cytoplasm, but a subpopulation of GSK-3β

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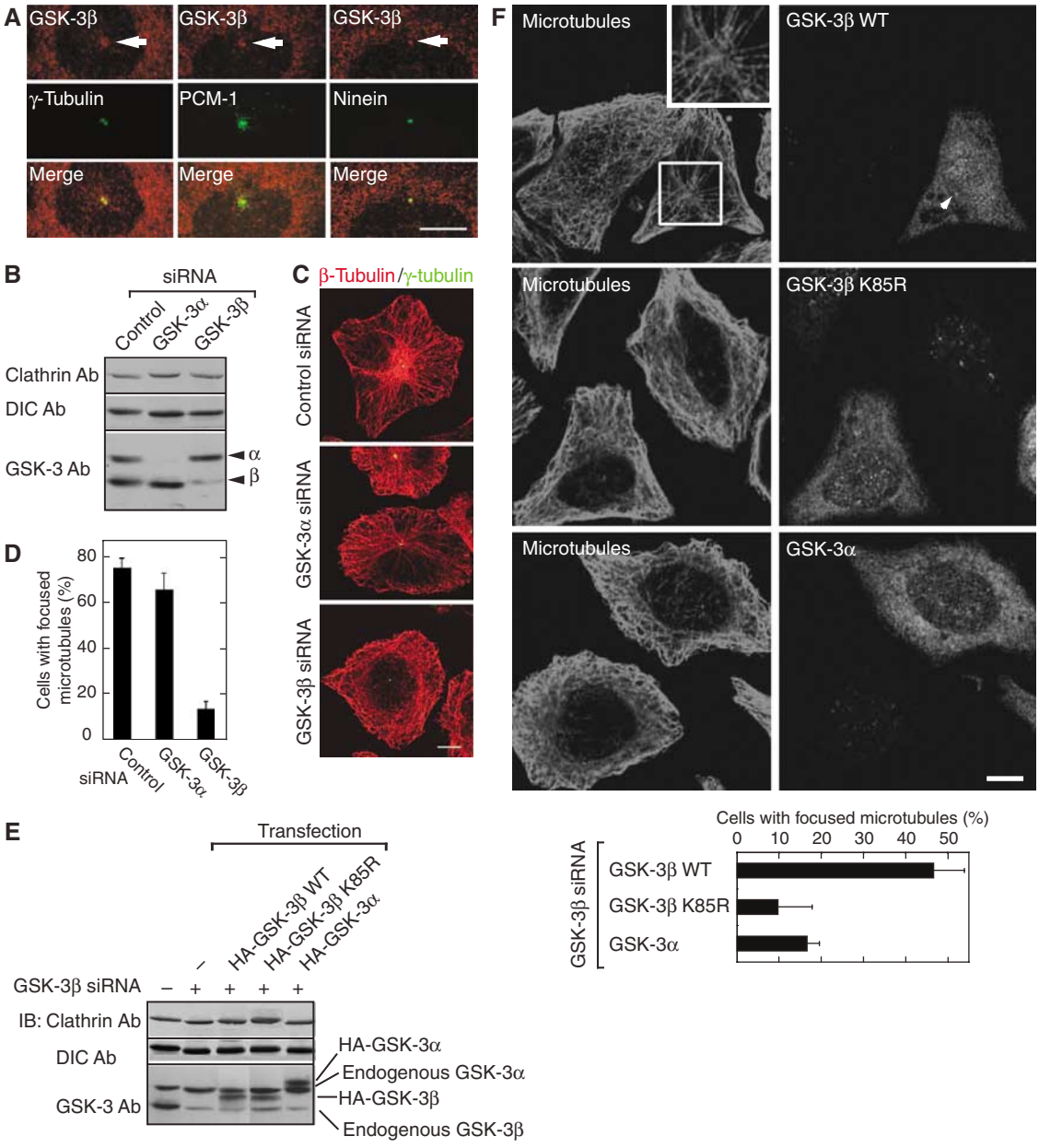

Figure 1 Involvement of GSK-3β in the organization of microtubules. (**A**) HeLa S3 cells were stained with the indicated antibodies. Arrows indicate centrosomal GSK-3β. (**B**) The lysates of HeLa S3 cells transfected with the indicated siRNA were probed with the indicated antibodies. Clathrin and DIC were used as loading controls. Ab, antibody. (**C**) HeLa S3 cells transfected with control, GSK-3α, or GSK-3β siRNA were stained with anti-β-tubulin (red) and anti-γ-tubulin (green) antibodies. (**D**) Cells with the radial array or the nonradial array microtubules were counted and the results shown are means ± s.d. of three independent experiments. (**E**) HA-GSK-3β, HA-GSK-3β^{K85R}, or HA-GSK-3α was expressed in GSK-3β knockdown cells. The lysates were probed with anti-GSK-3 antibody. GSK-3 Ab is an antibody that recognizes both GSK-3α and GSK-3β. As loading controls, the lysates were probed with anti-clathrin and anti-DIC antibodies. (**F**) HA-GSK-3β, HA-GSK-3β^{K85R}, or HA-GSK-3α was expressed in GSK-3β knockdown cells and the cells were stained with anti-β-tubulin and anti-HA antibodies. The insert in the upper right corner shows the magnified image of boxed area. Arrowhead indicates the centrosomal GSK-3β. Cell with focused microtubules were counted and the results shown are means ± s.d. of three independent experiments. Scale bar, 10 μm.

was located at the centrosome and it was colocalized with centrosomal proteins, including γ-tubulin, PCM-1, and ninein. Exogenously expressed HA-GSK-3β, which was recognized with anti-HA antibody, was also present with γ-tubulin (Supplementary Figure 1B). The similar findings with three different antibodies for GSK-3β strongly indicate the centrosomal localization of GSK-3β, and suggest the centrosomal function of GSK-3β.

To examine the roles of GSK-3 in the regulation of the minus end of microtubules, we performed knockdown of GSK-3α and GSK-3β by RNA interference (RNAi) in HeLa S3 cells (Figure 1B). In control cells, microtubules formed a radial array emerging from the centrosome and extending toward the cell periphery (Figure 1C). A large population of HeLa S3 cells showed this microtubule morphology (Figure 1D). To quantitate the cells where microtubules are attached to the centrosome, the integrated intensity of microtubules around the centrosome was divided by the intensity at the cell periphery. When the ratio was more than 1.5-fold, the cells were counted as 'cells with focused microtubules'.

Microtubules no longer appeared to radiate from the perinuclear focus in GSK-3β knockdown cells, although reduction of GSK-3α had little effect on the appearance of microtubules (Figure 1C and D). The phenotype in GSK-3β knockdown cells was restored by the expression of wild-type GSK-3β but not by a kinase-negative form of GSK-3β (GSK-3β^{K85R}) (Figure 1E and F). In these GSK-3β constructs, small interfering RNA (siRNA) target sites were silently mutated. Expression of GSK-3α caused little, if any, restoration of the focusing of microtubules to the centrosome. Loss of focused microtubule array was also observed in the cells treated with SB216763, a GSK-3 inhibitor (Supplementary Figure 1C).

We also examined the effect of knockdown of GSK-3 on the microtubule organization in U2OS cell, a well-characterized cell line that exhibits the clearly focused microtubules. GSK-3β knockdown U2OS cells showed the unfocused microtubule phenotype and the round-up shape by the GSK-3 depletion (data not shown). Therefore, the unfocused microtubule phenotype observed by the GSK-3β depletion was not specific for HeLa S3 cells.

Radial array formation of microtubules consists of at least two steps; the nucleation in and the anchoring to the centrosome. To dissect at which step(s) GSK-3β is involved in radial array formation of microtubules, microtubule regrowth experiments were performed. Microtubules were depolymerized by nocodazole, followed by washing out to allow regrowth of microtubules. In the initial phase of microtubule regrowth (Figure 2A, 2 and 6 min), short microtubules started to nucleate from the centrosome and create typical aster in both control and GSK-3β knockdown cells (Figure 2B). These results suggest that GSK-3β is not involved in the initial microtubule nucleation at the centrosome. Although microtubules were further elongated from the centrosome and organized into a radial array as time passed in control cells, GSK-3β knockdown cells decreased the centrosome-bound microtubules and the microtubule array resulted in the non-radial pattern (Figure 2A, 8 and 10 min, and B). Knockdown of GSK-3α did not affect the nucleation and anchoring of the microtubules (Figure 2B). Taken together, these results suggest that GSK-3β has a role in the anchoring but not in the nucleation of microtubules at the centrosome.

We also found that control cells contain more cytoplasmic microtubules than GSK-3β knockdown cells in the initial phase of microtubule regrowth. There are at least two possible mechanisms for the generation of cytoplasmic microtubules; one is due to the nucleation in the cytoplasm (Vorobjev *et al*, 1997) and the other is due to the microtubule release from the centrosome after the nucleation in the centrosome (Abal *et al*, 2002). If this microtubule appearance is solely dependent on the microtubule release from the centrosome, all microtubules would disappear from the centrosome in the later phase of regrowth. However, as seen in control cells, a subset of microtubules are bound to the centrosome. Therefore, after cytoplasmic microtubules are generated by these mechanisms, a subpopulation of microtubules may be anchored at the centrosome in control cells. At present, it is not clear whether knockdown of GSK-3 inhibits cytoplasmic nucleation of microtubules, or the microtubule release from the centrosome, or both.

Identification of BICD1 as a GSK-3β-binding protein

To clarify the mechanism by which GSK-3β mediates microtubule anchoring, we screened GSK-3β-binding protein(s) by

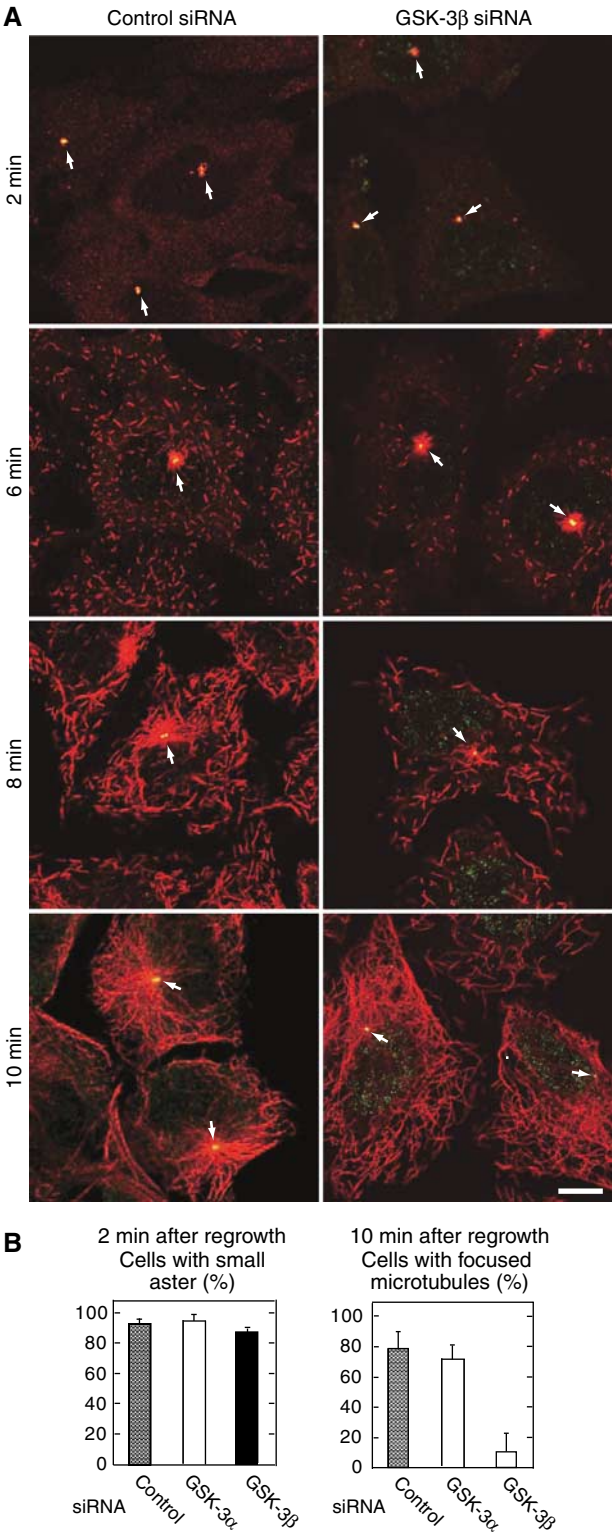

Figure 2 Involvement of GSK-3β in the anchoring of microtubules. (**A**) HeLa S3 cells transfected with control or GSK-3β siRNA were subjected to the microtubule regrowth assay and stained with anti-β-tubulin (red) and anti-γ tubulin (green) antibodies at 2, 6, 8 or 10 min after regrowth. The arrows indicate the positions of centrosome. Scale bar, 10 μm. (**B**) Cells with small asters at 2 min and cells with focused microtubules at 10 min were counted (at least 300 cells were evaluated for each experimental group) and the results shown (right panels) are means ± s.d. of three independent experiments. Several short microtubule fragments were observed in some cells at 2 min after regrowth.

yeast two-hybrid screening and isolated BICD1. BICD1 is a human homologue of *Drosophila* BICD (Suter *et al*, 1989) and there are two homologues in mammals, BICD1 and BICD2 (Hoogenraad *et al*, 2001). BICD proteins consist of three coiled-coiled domains (Figure 3A) and are involved in dynein-mediated minus end-directed transport from the Golgi apparatus to the endoplasmic reticulum (ER) (Matanis *et al*, 2002; Hoogenraad *et al*, 2003).

Immunoprecipitation analyses showed that GSK-3β and BICD1 form a complex at the endogenous level in HeLa S3 cells (Figure 3B). GSK-3β also formed a complex with BICD2 (data not shown). Although GSK-3α also interacted with BICD1, the amount of BICD1 co-precipitated with GSK-3α was lower than that with GSK-3β (Figure 3B). *In vitro* binding studies using recombinant proteins demonstrated that His_6-GSK-3β bound directly to GST-BICD1 (Figure 3C). To identify which region of BICD1 is important for the binding to GSK-3β, various deletion mutants of Myc-BICD1 were expressed in COS cells (Figure 3D). In addition to Myc-BICD1 (wild type), Myc-BICD1-(380–701) and Myc-BICD1-(437–617) formed a complex with GSK-3β, but Myc-BICD1-(1–435) did not. HA-GSK-3β kinase-inactive mutants (K85M and K85R) did not form a complex with Myc-BICD1 under the conditions in which wild-type GSK-3β and a constitutively active GSK-3β mutant (S9A) did (Figure 3E). These results indicate that amino-acid region 437–617 of BICD1 and the kinase activity of GSK-3 are necessary for the formation of a complex between BICD1 and GSK-3β in intact cells.

Consistent with previously reported observations (Hoogenraad *et al*, 2001), BICD1 exhibited a perinuclear and punctate cytoplasmic distribution in HeLa S3 cells fixed with paraformaldehyde, and it overlapped with a Golgi marker, γ-adaptin (Supplementary Figure 2A). In addition, BICD1 was also present with γ-tubulin, $p150^{glued}$ (a large subunit of dynactin), and GSK-3β at the centrosome in cold methanol-fixed cells (Figure 4A). Another anti-BICD1 antibodies confirmed the colocalization of BICD1 with γ-tubulin (data not shown). BICD2 is also localized to the centrosome (Supplementary Figure 2B).

To examine the presence of GSK-3β and BICD1 in the centrosome biochemically, we isolated the centrosome from CHO cells by the discontinuous sucrose-density gradient (Mitchison and Kirschner, 1986). The centrosome, as indicated by the presence of γ-tubulin, was located to the inter-

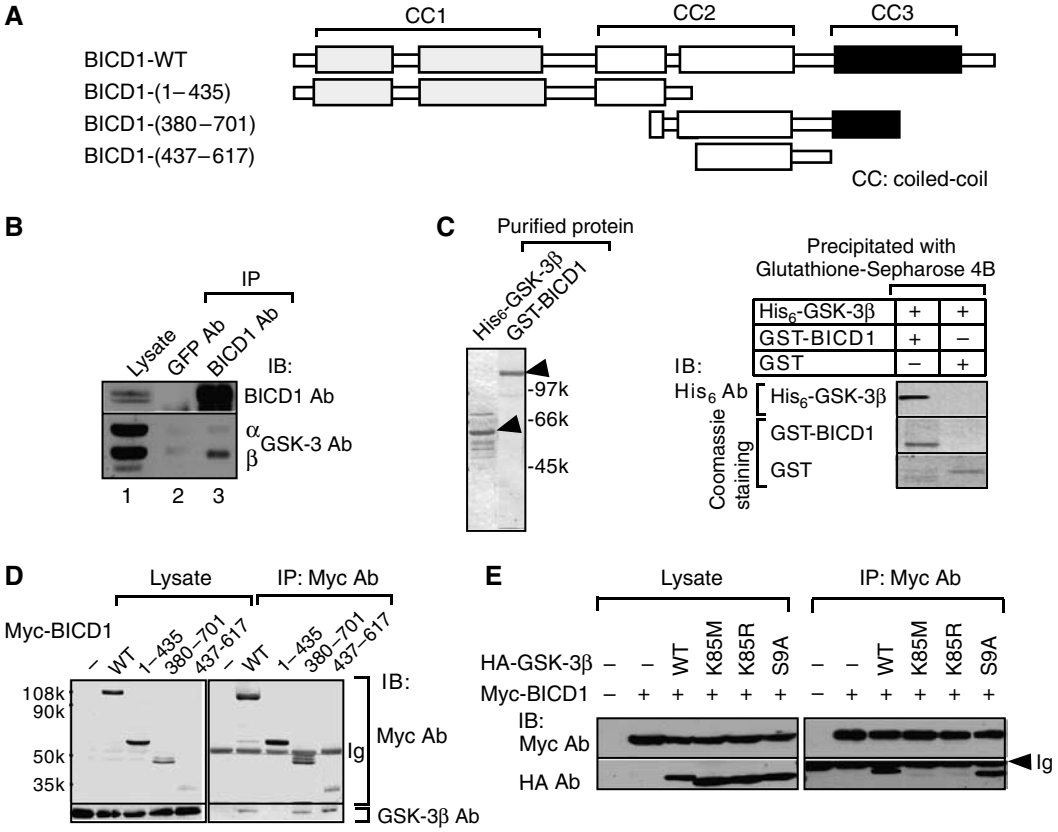

Figure 3 Identification of BICD1 as a GSK-3β-binding protein. (**A**) Schematic representation of the deletion mutants of BICD1 used in this study. CC, coiled-coiled domain. (**B**) Lysates of HeLa S3 cells (lane 1) were immunoprecipitated with anti-GFP (lane 2) or anti-BICD1 (lane 3) antibody, and the immunoprecipitates were probed with the indicated antibodies. IP, immunoprecipitation; IB, immunoblotting; Ab, antibody. (**C**) Recombinant His_6-GSK-3β and GST-BICD1 (0.5 μg of protein) were stained with Coomassie brilliant blue (left panel). After His_6-GSK-3β was incubated with GST-BICD1 or GST, GST-BICD1 and GST were precipitated with glutathione-Sepharose and then the precipitates were probed with anti-His_6 antibody (right panel). (**D**) Lysates of COS cells expressing the deletion mutants of Myc-BICD1 were probed with anti-Myc or anti-GSK-3β antibody (left panel). The same lysates were immunoprecipitated with anti-Myc antibody, and the immunoprecipitates were probed with anti-Myc or anti-GSK-3β antibody (right panel). (**E**) Lysates of HEK293T cells expressing Myc-BICD1 and HA-GSK-3β mutants were probed with anti-Myc or anti-HA antibody (left panel). The same lysates were immunoprecipitated with anti-Myc antibody, and the immunoprecipitates were probed with anti-Myc or anti-HA antibody (right panels). An arrowhead indicates immunoglobulin (Ig) detected nonspecifically by the secondary antibody for anti-HA antibody.

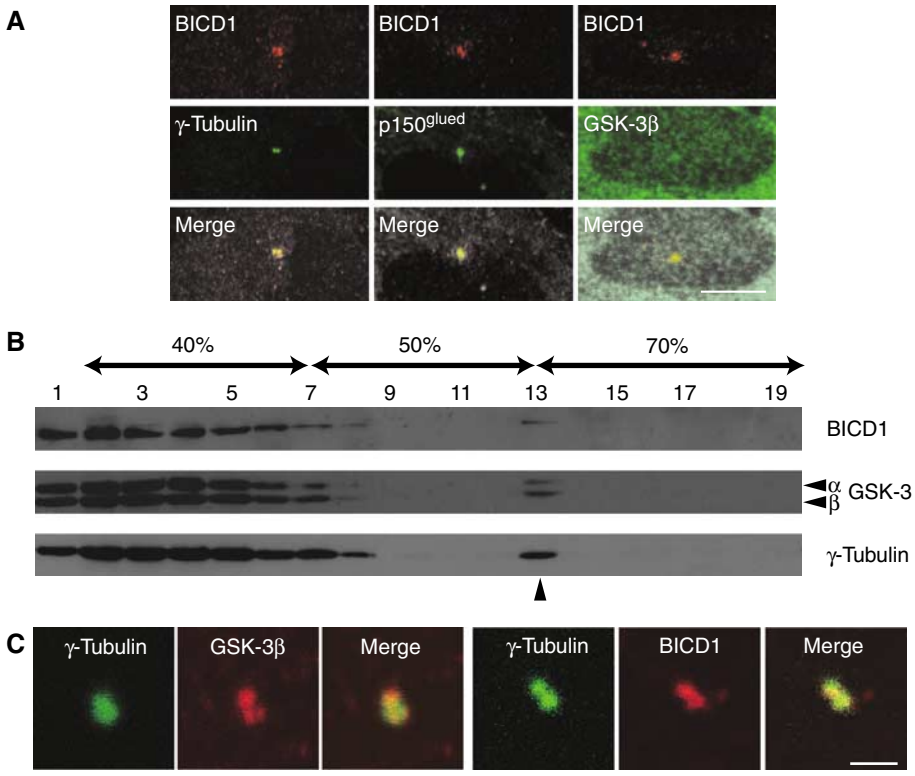

Figure 4 Colocalization of GSK-3β and BICD at the centrosome. (**A**) HeLa S3 cells were stained with anti-BICD1, anti-γ-tubulin, anti-p150^{glued}, or anti-GSK-3β antibody. Scale bar, 10 μm. (**B**) The lysates from CHO cells were fractionated by the sucrose gradient and probed with the indicated antibodies. The arrowhead indicates the centrosome-enriched fraction. (**C**) Isolated centrosomes were stained with the indicated antibodies. Scale bar, 2 μm.

face between 50 and 70% sucrose gradient (Figure 4B). In addition, GSK-3β and BICD1 were also cosedimented with the γ-tubulin, suggesting that GSK-3β and BICD1 are the integral centrosomal components. GSK-3α was also observed in the centrosomal fractions (Figure 4B). Furthermore, we spotted the centrosome fraction on the cover glass and the fraction was stained with anti-GSK-3β, BICD1, and γ-tubulin antibodies. GSK-3β was localized with a part of the γ-tubulin spot and also an additional portion of the centrosome (Figure 4C). BICD1 was almost overlapped with γ-tubulin (Figure 4C). Although GSK-3β and γ-tubulin may appear not to be overlapped exactly, 74.6% ($n=75$) and 76.6% ($n=60$) of spots stained with anti-γ-tubulin antibody were positive for GSK-3β and BICD1, respectively. These results strongly suggest that GSK-3β and BICD1 are integrated in the centrosome and that BICD is involved in the centrosomal functions.

Involvement of BICD in the anchoring of microtubules

To examine the new function of BICD, and the protein levels of BICD1 or BICD2 were decreased by RNAi (Figure 5A). BICD1 and BICD2 disappeared from the centrosome by the RNAi treatment (Supplementary Figure 2B). Reduction of either BICD1 or BICD2 induced weak defects in the radial array formation of microtubules (data not shown) and double knockdown of BICD1 and BICD2 (BICD1/2) showed more robust phenotypes (Figure 5B), suggesting that BICD1 and BICD2 compensate the function of each other. However, γ-tubulin and p150^{glued} were detected at the centrosome in BICD1/2 double knockdown cells (Figure 5B and data not shown). Microtubule regrowth assay demonstrated that the ability of microtubules to anchor to the centrosome is lost but nucleation is observed at the centrosome in BICD1/2 double knockdown cells (Figure 5C). The similar phenotypes in GSK-3β and BICD knockdown cells suggest the functional interaction of GSK-3β and BICD.

As Rab6 small GTPase is known to be involved in the Golgi–ER trafficking through binding to BICD (Matanis *et al*, 2002), we examined whether Rab6 also has a role in microtubule organization. Microtubules anchored at the centrosome were seen in Rab6 knockdown cells, although a subset of microtubules were released and distributed along the cell periphery when compared with control cells (Supplementary Figure 3A and B). These observations were different from those seen in BICD1/2 knockdown cells (see Figure 5B). As it has been demonstrated that the Golgi apparatus functions as a microtubule anchorage site (Rios *et al*, 2004), partially released microtubules may have been caused by the disruption of the Golgi apparatus in Rab6 knockdown cells. Knockdown of Rab6 induced severe Golgi disruption as previously reported (Young *et al*, 2005), whereas knockdown of BICD1/2 affected the Golgi morphology to a lesser extent (Supplementary Figure 3C). Taken together, these results suggest that BICD has a specific role in the microtubule anchoring to the centrosome in a Rab6-independent manner.

Functional interaction between GSK-3β and BICD1

BICD2 has been shown to interact with dynein and dynactin (Hoogenraad *et al*, 2001). Endogenous dynein intermediate chain (DIC), p150^{glued}, and p50^{dynamitin} (another dynactin subunit) were co-precipitated with BICD1 as well as GSK-3β (Figure 6A). When the protein levels of GSK-3β were reduced

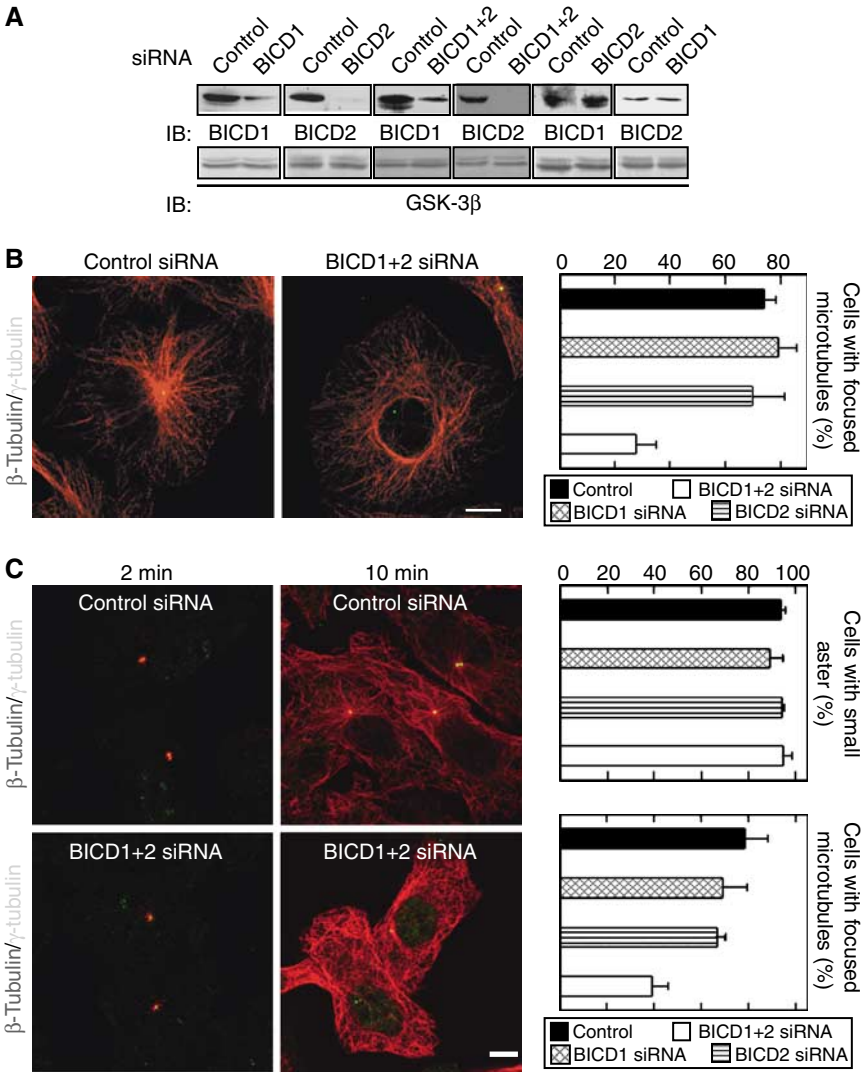

Figure 5 Involvement of BICD in the anchoring of microtubules. (**A**) The lysates of HeLa S3 cells transfected with control, BICD1, and/or BICD2 siRNA were probed with anti-BICD1, anti-BICD2, or anti-GSK-3β antibodies. (**B**) HeLa S3 cells transfected with control or BICD1 and BICD2 siRNA were stained with anti-β-tubulin (red) and γ-tubulin (green) antibodies. Cells with the radial array or the nonradial array microtubules were counted and the results shown are means ± s.d. of three independent experiments (right panels). (**C**) HeLa S3 cells transfected with control (upper panels) or BICD1 and BICD2 (lower panels) siRNA were subjected to the microtubule regrowth assay and fixed at 2 or 10 min after regrowth. The cells were stained with anti-β-tubulin (red) and γ-tubulin (green) antibodies. Cells with small asters at 2 min and cells with focused microtubules at 10 min were counted and the results shown are means ± s.d. of three independent experiments (right panels). Scale bars, 10 μm.

by RNAi or when the cells were treated with SB216763, the interaction of BICD1 with DIC and GSK-3β was impaired but BICD1 still bound to $p150^{glued}$ and $p50^{dynamitin}$ (Figure 6A). Therefore, the kinase activity of GSK-3 is required for the formation of a complex between BICD1 and dynein. Furthermore, endogenous BICD1 was not present at the centrosome in the GSK-3β knockdown cells and SB216763-treated cells (Figure 6B). The centrosomal localization of γ-tubulin and DIC was not affected in these cells (Figure 6B and data not shown). As it has been shown that BICD2 acts as an accessory factor for the dynein motor (Hoogenraad *et al*, 2001; Matanis *et al*, 2002) and that the dynein–dynactin complex is implicated in anchoring function at the centrosome (Quintyne *et al*, 1999), these results prompted us to examine the cooperative functions of BICD1 and the dynein and dynactin complex in the minus ends of microtubules.

To examine whether GSK-3β-regulated interaction of BICD1 with dynein is involved in the anchoring of microtubules to the centrosome, we generated a fusion construct in which DIC was fused to the N-terminus of BICD1 (HA-DIC-BICD1) and introduced into GSK-3β knockdown cells. HA-DIC-BICD1, HA-BICD1, and HA-DIC showed diffuse cytosolic distribution and centrosomal localization when these proteins were expressed in control HeLa S3 cells (Supplementary Figure 4A). Localization of these expressed proteins at the centrosome was confirmed by colocalization with centrin 3 (Supplementary Figure 4B). Expression of these proteins did not affect the radial array formation of microtubules (Supplementary Figure 4A). When HA-BICD1 was expressed in GSK-3β knockdown cells, it was observed diffusely with dots and not concentrated to the perinuclear region (Figure 6C). HA-DIC1 showed diffuse cytosolic distribution and centrosomal localization in GSK-3β knockdown cells

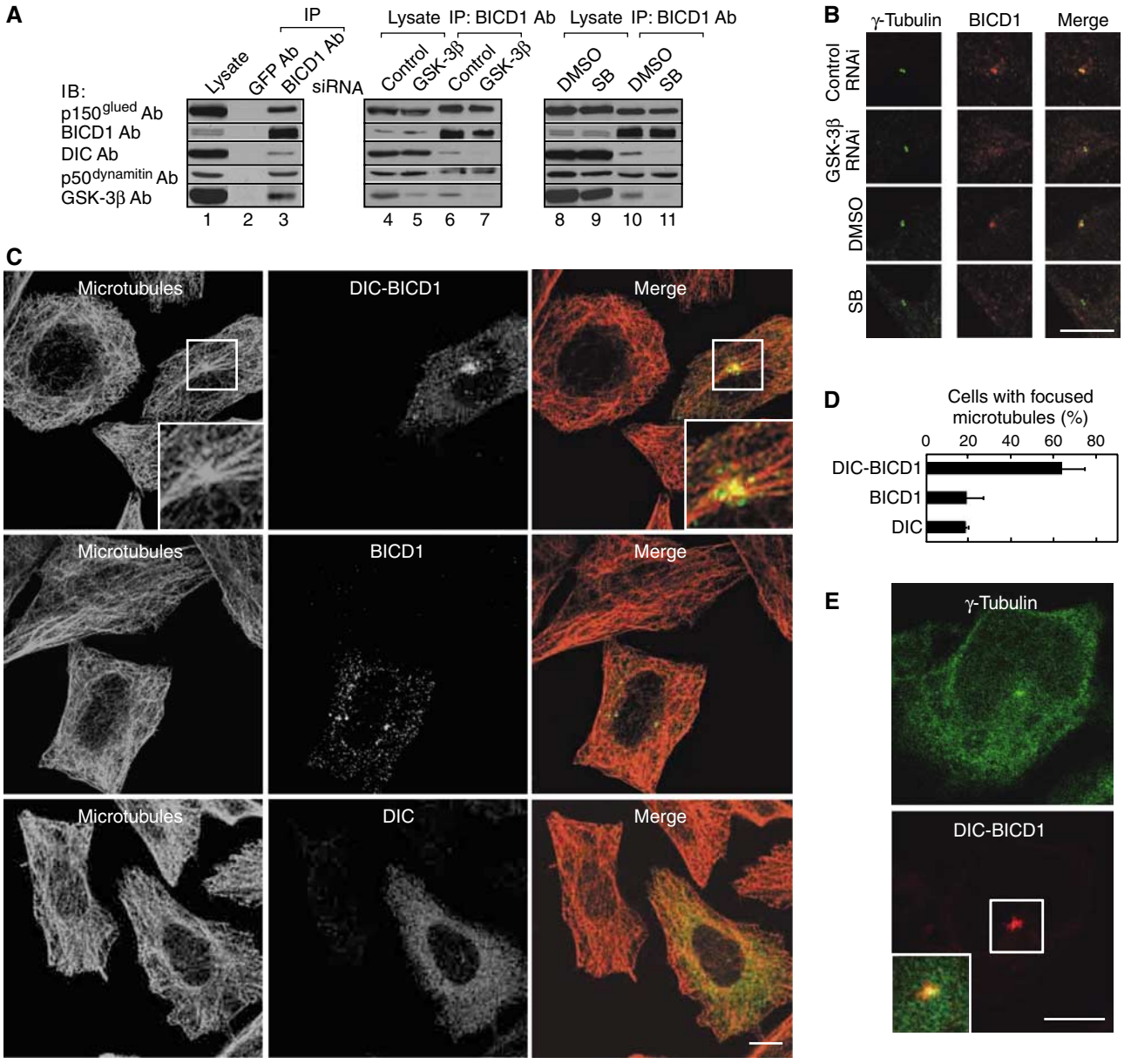

Figure 6 Functional interaction between GSK-3β and BICD1. (**A**) Lysates of HeLa S3 cells (lane 1) were immunoprecipitated with anti-GFP (lane 2) or anti-BICD1 (lane 3) antibody. Lysates of HeLa S3 cells transfected with control siRNA (lanes 4 and 6) or GSK-3β siRNA (lanes 5 and 7), and lysates of HeLa S3 cells treated with either DMSO (lanes 8 and 10) or 30 μM SB216763 (lanes 9 and 11) were immunoprecipitated with anti-BICD1 antibody. The immunoprecipitates were probed with the indicated antibodies. (**B**) HeLa S3 cells transfected with GSK-3β siRNA or treated with 30 μM SB216763 were stained with the indicated antibodies. (**C**) HA-DIC-BICD1, HA-BICD1, or HA-DIC was expressed in GSK-3β knockdown HeLa S3 cells. The inserts in the lower right corner of upper panels show the magnified image of boxed area. In merged images, β-tubulin and expressed proteins were shown as red and green, respectively. (**D**) Cells with focused microtubules in Figure 3C were counted (at least 70 cells were evaluated for each experimental group) and the results shown are means ± s.d. of three independent experiments. (**E**) HA-DIC-BICD1 was expressed in GSK-3β knockdown HeLa S3 cells and the cells were stained anti-HA and anti-γ-tubulin antibodies. The insert shows the merged image of the boxed area. Scale bars, 10 μm.

(Figure 6C). Unfocused microtubules in these cells were not affected by the expression of HA-BICD1 or HA-DIC (Figure 6C and D). In contrast, expression of HA-DIC-BICD1 in GSK-3β knockdown cells significantly restored unfocused microtubules (Figure 6C and D). Furthermore, HA-DIC-BICD1 was colocalized with γ-tubulin, indicating that this fusion protein was located to the centrosome (Figure 6E). These results suggest that the stabilization of the BICD–dynein complex by GSK-3β is important for the maintenance of the focused radial array of microtubules.

Phosphorylation of BICD1 by GSK-3β is essential for the radial array formation of microtubules

Endogenous BICD1 was immunoprecipitated with anti-BICD1 and incubated with calf intestinal alkaline phosphatase (CIAP). The slowly migrating band of endogenous BICD1 on SDS–PAGE disappeared by treatment with CIAP, suggesting that BICD1 is phosphorylated (Figure 7B). When Myc-BICD1 was expressed alone, it migrated as a broad band on SDS–PAGE. The relative intensity of the upper bands of Myc-BICD1 was increased when Myc-BICD1 was coexpressed with

wild-type GSK-3β and decreased when it was coexpressed with GSK-3β^{K85R} (Figure 7B). Therefore, GSK-3β may be involved in the phosphorylation of BICD1. The sequence of S/TXXXS/T is known to be a consensus sequence of the GSK-3β phosphorylation site. Between amino acids 570 and 609 of BICD1, there are some possible phosphorylation sites (Figure 7A). All serine and threonine residues in these possible phosphorylation sites were substituted with alanine (Figure 7A: 3A, 570/574/578A and 7A, 585/589/593/597/601/605/609A). Although Myc-BICD1-3A exhibited the same

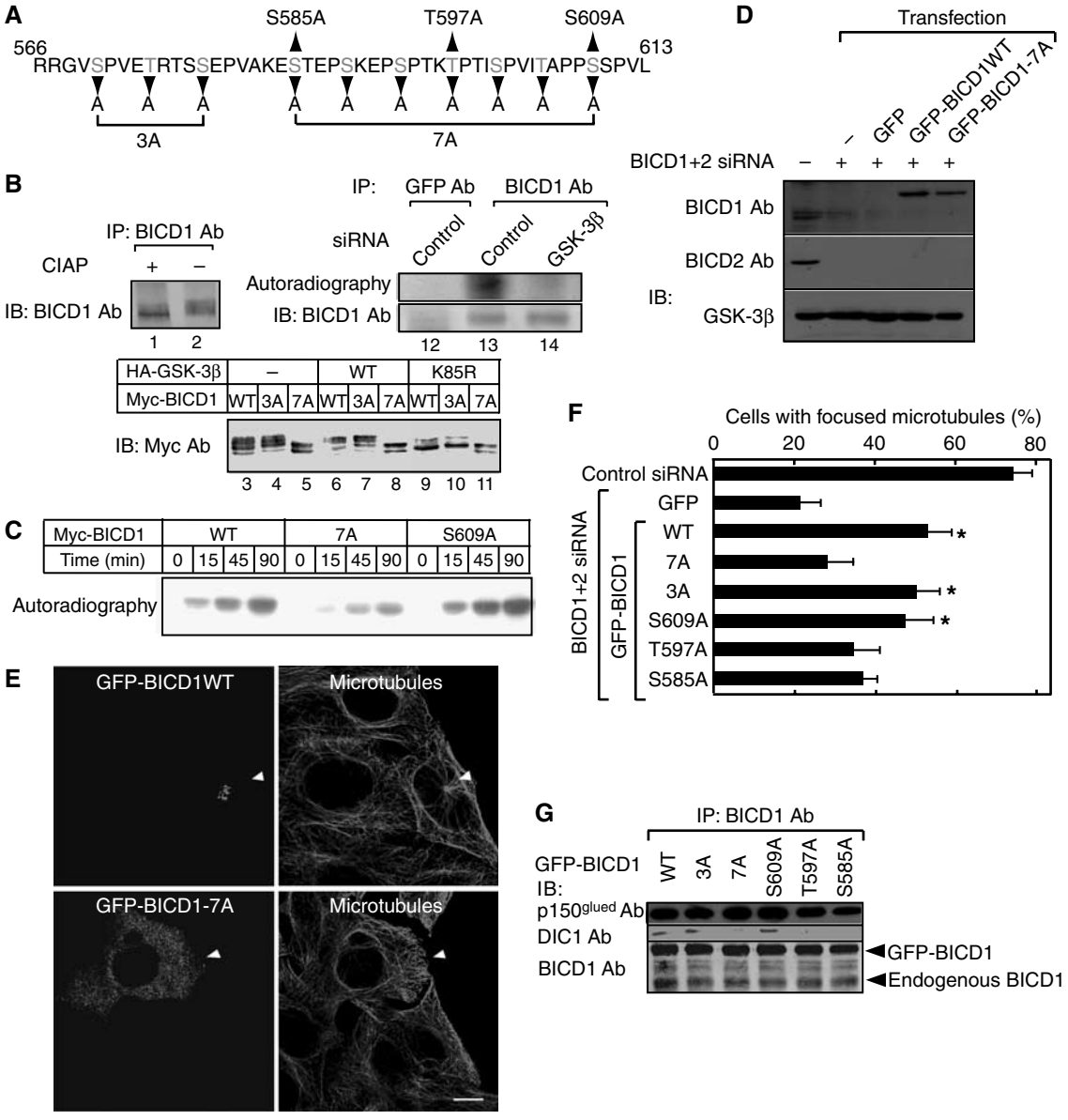

Figure 7 Involvement of the phosphorylation of BICD1 by GSK-3β in the radial array formation of microtubules. (**A**) Putative sites of phosphorylation of BICD1 by GSK-3β. Various mutants of BICD1 were generated (S585A, T597A, S609A, 570/574/578A (3A), and 585/589/593/597/601/605/609A (7A)). (**B**) Lysates of HeLa S3 cells were immunoprecipitated with anti-BICD1 antibody (lanes 1 and 2), and the immunoprecipitates were incubated with (lane 1) or without (lane 2) alkaline phosphatase. Lysates of HEK293T cells expressing Myc-BICD1 (lanes 3, 6, and 9), Myc-BICD1-3A (lanes 4, 7, and 10), or Myc-BICD1-7A (lanes 5, 8 and 11) with HA-GSK-3β (lanes 6–8) or HA-GSK-3β^{K85R} (lanes 9–11) were probed with anti-Myc antibody. HeLa S3 cells transfected with control (lanes 12 and 13) or GSK-3β (lane 14) siRNA were metabolically labeled with ^{32}P (3.7 MBq) for 4 h. Lysates of the cells were immunoprecipitated with control (lane 12) or anti-BICD1 antibody (lanes 13 and 14). The immunoprecipitates were probed with anti-BICD1 antibody and subjected to autoradiography. (**C**) Myc-BICD1, Myc-BICD1-7A, or Myc-BICD1^{S609A} was immunoprecipitated from HEK293T cells and the immunoprecipitates were incubated with purified His$_6$-GSK-3β in the presence of [γ-^{32}P]ATP for the indicated periods of time. The samples were subjected to autoradiography. (**D**) Sixty hours after HeLa S3 cells were transfected with BICD1 and BICD2 siRNA, GFP, GFP-BICD1, or GFP-BICD-7A was expressed in the cells for 24 h and then the lysates were probed with the indicated antibodies. (**E**) The cells prepared in (D) were stained with anti-GFP or anti-β-tubulin antibody. Arrowheads indicate the cells expressing GFP-BICD1 or GFP-BICD-7A. Scale bar, 10 μm. (**F**) Cells with focused microtubules were counted (at least 200 cells were evaluated for each experimental group). The results shown are means ± s.d. of at least three independent experiments. Asterisks indicate that the difference between GFP and GFP-BICD WT or its mutants (3A and T609A) expressing cells were statistically significant ($P<0.005$). Bars without asterisks are not significant. (**G**) Lysates of HeLa S3 cells expressing GFP-BICD1, GFP-BICD1-3A, GFP-BICD1-7A, GFP-BICD1^{S609A}, GFP-BICD1^{T597A}, or GFP-BICD1^{S585A} were immunoprecipitated with anti-BICD1 antibody, and the immunoprecipitates were probed with the indicated antibodies. The results shown are representatives of three independent experiments.

mobility shift as Myc-BICD1, Myc-BICD1-7A showed a reduced mobility shift (Figure 7B). Neither wild-type GSK-3β nor GSK-3β^{K85R} affected the mobility of this mutant (Figure 7B). The extent of the phosphorylation level of BICD1 in GSK-3β knockdown cells metabolically labeled with ^{32}P was reduced as compared with that of the control cells (Figure 7B). Purified GSK-3β phosphorylated Myc-BICD1 in a time-dependent manner *in vitro* but Myc-BICD1-7A was phosphorylated to a lesser degree (Figure 7C). In the absence of purified GSK-3β, Myc-BICD1 was not phosphorylated (data not shown). Therefore, the serine and threonine residues in the region between amino acids 585 and 609 could be GSK-3β phosphorylation sites.

To clarify the function of GSK-3β-dependent phosphorylation of BICD1, BICD1 mutants, in which siRNA target sequences were silently mutated, were expressed in BICD1/2 double knockdown cells (Figure 7D). GFP-BICD1 (Figure 7E and F) and GFP-BICD1-3A (data not shown) located to the centrosome, and microtubules radiated from the place where GFP-BICD1 was present. In contrast, GFP-BICD1-7A was distributed diffusely and did not restore the radial array formation of microtubules (Figure 7E and F). Furthermore, we mutated Ser585, Thr597, or Ser609 to Ala in BICD1 (S585A, T597A, and S609A) (Figure 7A). Among these mutants, GFP-BICD1-3A and GFP-BICD1^{S609A} formed a complex with endogenous DIC as well as GFP-BICD1, but GFP-BICD1-7A, GFP-BICD1^{S585A}, and GFP-BICD1^{T597A} did not (Figure 7G). Consistent with these results, GFP-BICD1^{S585A} and GFP-BICD1^{T597A}, but not GFP-BICD1^{S609A}, lost the ability to restore the radial array formation of microtubules (Figure 7F). Therefore, at least Ser585 and Thr597 in BICD1 are important phosphorylation sites for BICD1 to exert its functions, and GSK-3β-dependent phosphorylation is required for the interaction of BICD1 with dynein. Consistent with this idea, Myc-BICD1^{S609A} was phosphorylated by purified GSK-3β to an extent similar to Myc-BICD1 (Figure 7C), but the phosphorylation of Myc-BICD1^{T597A} was reduced to a lesser degree (data not shown).

Centrosomal localization of ninein mediated by BICD1 and GSK-3β

Centrosomal proteins, such as ninein, PCM-1, and centrin 3, play an important role in the positioning and anchoring of the microtubule minus ends, and their localization is known to be regulated by the dynein–dynactin system (Dammermann and Merdes, 2002). BICD1 binds to the cargo via its C-terminal domain and to the dynein motor via its N-terminal domain (Hoogenraad *et al*, 2001). We hypothesized that BICD1 transports some centrosomal proteins to the centrosome in a dynein-dependent manner. Although ninein, PCM-1, and centrin 3 localized to the centrosome in control cells, ninein was not concentrated at the centrosome in GSK-3β knockdown or BICD1/2 double knockdown cells (Figure 8A). When the amounts of ninein at the centrosome were quantified, they were indeed decreased in GSK-3β or BICD1/2 knockdown cells as compared with control cells (Figure 8B). PCM-1 localized to the pericentrosomal area in BICD1/2 double knockdown cells and partially colocalized with γ-tubulin, but it was not concentrated to the centrosome in GSK-3β knockdown cells (Figure 8A). In contrast, knockdown of BICD1/2 or GSK-3β had no impact on the localization of γ-tubulin or centrin 3 at the centrosome (Figure 8A).

When the lysates of HEK293T cells expressing Myc-BICD1 and GFP-ninein were immunoprecipitated with anti-Myc antibody, GFP-ninein was detected in the Myc-BICD1 immune complex (Figure 8C). Furthermore, GFP-ninein formed a complex with Myc-BICD1-3A and Myc-BICD1-7A (Figure 8C). Microtubules freely assembled in the cytoplasm are induced by taxol treatment and their minus ends are not attached to the centrosome (De Brabander *et al*, 1981), but they contain centrosomal proteins including anchoring proteins (Bornens, 2002). By the treatment of HeLa S3 cells expressing GFP-ninein with taxol, the centrosomal microtubules disappeared and GFP-ninein was observed at the cell periphery where BICD1 was also colocalized (Figure 8D). When HA-DIC-BICD1 was expressed in GSK-3β knockdown cells, ninein was colocalized with this fusion protein (Figure 8E). Taken together with the observations that HA-DIC-BICD1 was located to the centrosome (see Figure 6E), these results indicate that expression of HA-DIC-BICD1 induces the recruitment of ninein to the centrosome in GSK-3β knockdown cells.

As previously reported (Dammermann and Merdes, 2002), focused microtubules were hardly observed in ninein knockdown cells (Supplementary Figure 5A and B). Expression of HA-DIC-BICD1 in ninein knockdown cells did not rescue this phenotype (Supplementary Figure 5C). Taken together, these observations suggest that BICD could bind to ninein independently of the phosphorylation state of BICD by GSK-3β and to the dynein motor in cooperation with GSK-3β, and thereby, transport ninein to the centrosome.

Discussion

New roles of GSK-3β in centrosome through BICD

During the course of analyzing the multiple functions of GSK-3β, we found that microtubules do not radiate from the centrosome in GSK-3β knockdown cells. GSK-3β has already been shown to regulate microtubule dynamics by phosphorylating several substrates including MAPs and the plus-end tracking proteins (+TIPs), such as APC and CLASP (Akhmanova *et al*, 2001; Cohen and Frame, 2001; Jope and Johnson, 2004). In addition, GSK-3β is present at the plus ends of microtubules and mediates cell polarization and protrusion through microtubules (Etienne-Manneville and Hall, 2003). As the phosphorylation of these substrates by GSK-3β influences their association with microtubules and local microtubule stability, the regulation of +TIPs by GSK-3β may be involved in the radial array formation of microtubules. However, in this study, we focused on a new aspect of roles of GSK-3β in the centrosome because GSK-3β was present there. It has been reported that GSK-3 interacts with microtubules during mitosis and accumulates at the centrosomes and spindle poles in mammalian cells (Wakefield *et al*, 2003) and that Shaggy, the *Drosophila* homologue of GSK-3β, becomes enriched in the centrosome throughout mitosis (Bobinnec *et al*, 2006). However, the roles of GSK-3β in the centrosome have yet to be clarified. We speculated that GSK-3β has a role in the regulation of the transport of the proteins necessary for the anchoring of microtubules to the centrosome and/or in the microtubule anchoring itself.

To prove this idea, we paid an attention to BICD among the GSK-3β-binding proteins, which are isolated by yeast

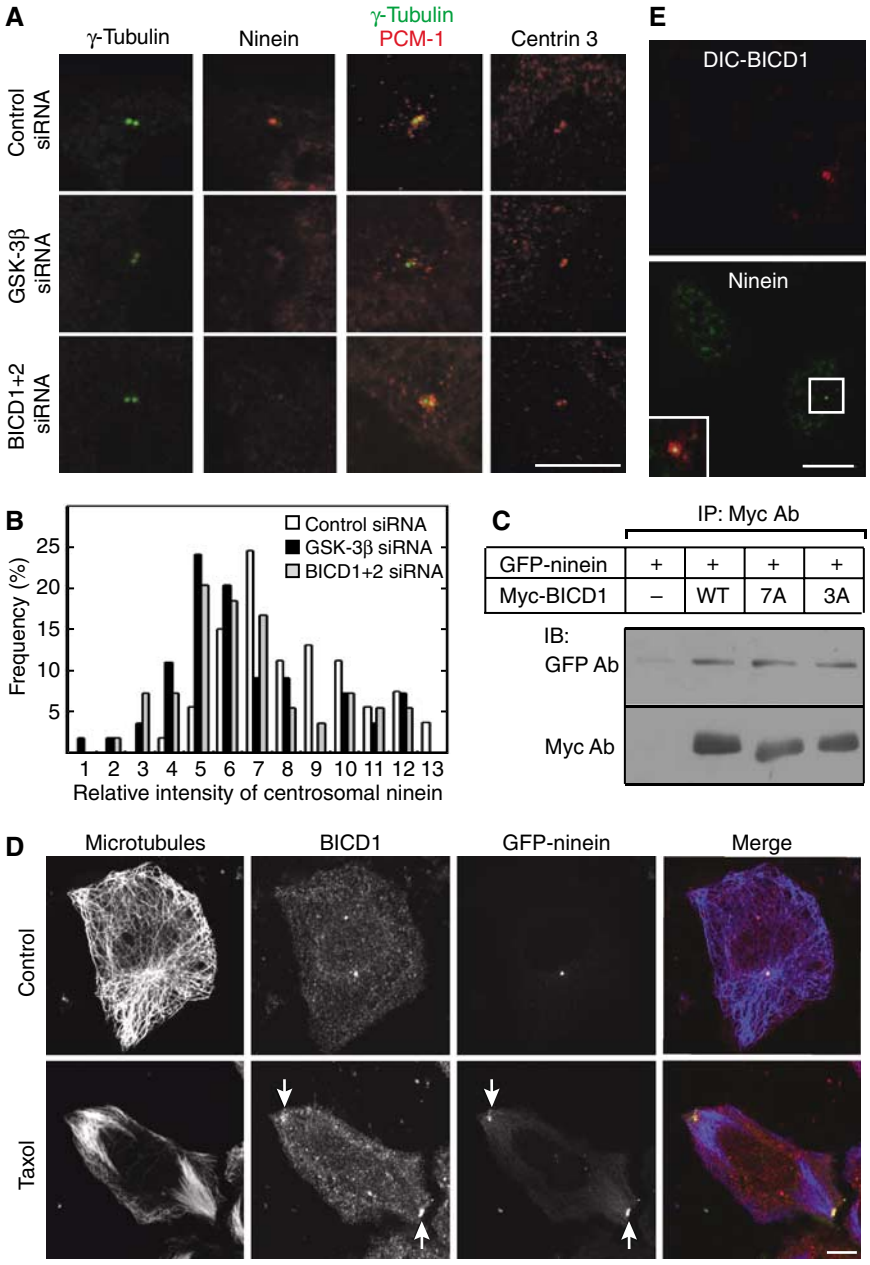

Figure 8 Centrosomal localization of ninein mediated by GSK-3β and BICD1. (**A**) HeLa S3 cells transfected with control, GSK-3β, or BICD1 and BICD2 siRNA were stained with the indicated antibodies. (**B**) The fluorescence intensity (as indicated by 1–13) of encircled region (2 μm diameter), where the centrosome is located, in siRNA-transfected HeLa S3 cells (200 cells) was shown as frequency histogram. Fluorescence intensity was measured by MetaMorph software. The intensity of ninein at the centrosome in GSK-3β or BICD1/2 knockdown cells was statistically lower than that in control cells ($P<0.005$, Kolmogorov–Smirnov test). (**C**) The lysates of HEK293T cells expressing Myc-BICD1 and mutants and GFP-ninein were immunoprecipitated with anti-Myc antibody, and the immunoprecipitates were probed with anti-Myc or anti-HA antibody. (**D**) HeLa S3 cells expressing GFP-ninein were treated with or without 1 μM taxol for 14 h and stained with anti-β-tubulin and anti-BICD1 antibodies. GFP-ninein was viewed directly with a microscope. Arrows indicate the colocalization of BICD1 and ninein at the microtubule minus ends. Colocalization of microtubules (blue), BICD1 (red), and ninein (green) was shown as white dots in merged pictures. (**E**) HA-DIC-BICD1 was expressed in GSK-3β knockdown HeLa S3 cells and the cells were stained with anti-HA and anti-ninein antibodies. The insert shows the merged image of the boxed area. Scale bars, 10 μm.

two-hybrid screening. In addition to a role of BICD in the Golgi–ER trafficking (Matanis *et al*, 2002), we found that BICD regulates the anchoring of microtubules to the centrosome, as BICD1/2 knockdown or overexpression of the BICD1 deletion mutants (data not shown) induced microtubule unfocusing. Therefore, it is conceivable that GSK-3β and BICD share common roles in at least the anchoring of microtubules to the centrosome.

Regulation of functions of BICD by GSK-3β-dependent phosphorylation

We found that BICD is a phosphoprotein and that this phosphorylation could be regulated by GSK-3β. Although the phosphorylation state of BICD1 was affected by GSK-3β in intact cells, the degree of phosphorylation of BICD1 by GSK-3β *in vitro* was low. Many substrates of GSK-3 require prior phosphorylation by some other protein kinase (Plyte

et al, 1992; Cohen and Frame, 2001). Prior phosphorylation by some kinase that remains to be identified might result in more efficient phosphorylation of BICD1 by GSK-3β.

We showed that BICD1 mutants, in which the serine or threonine residues in the possible phosphorylation sites by GSK-3β are changed to alanine, lose the ability to restore the microtubule unfocusing in BICD1/2 double knockdown cells. These results indicate that the phosphorylation of BICD1, most probably by GSK-3β, is important for the anchoring of microtubules to the centrosome. Our mutational analyses suggested that Ser585 and Thr597 but not Ser609 are phosphorylation sites. Given the processive nature of GSK-3 phosphorylation, the phosphorylation of the C-terminal site (Ser609) fits the idea that a C-terminal phosphoserine or phosphothreonine is important for the substrate specificity of GSK-3. However, this is not the case for BICD1 and the presence of the similar substrates has been reported (Holmes *et al*, 1986). Casein kinase-II (CK-II) phosphorylates glycogen synthase and inhibitor-2 and thereby potentiates phosphorylation by GSK-3, although the distance between serine and/or threonine residues phosphorylated by CK-II and GSK-3 is clearly variable and there are serine and threonine residues that are not phosphorylated between the phosphorylation sites by CK-II and GSK-3.

The kinase activity of GSK-3β was necessary for the formation of a complex between BICD1 and DIC, and BICD1 mutants in which the possible phosphorylation sites by GSK-3β are mutated, lost the ability to bind to DIC. These results suggest that GSK-3β-dependent phosphorylation of BICD1 is important for the transport of BICD1 by the dynein–dynactin complex. Knockdown of GSK-3β induced the loss of anchoring of microtubules to the centrosome, and it also caused the dissociation of BICD from the dynein complex. These two phenotypes in GSK-3β knockdown cells were linked, because the radial formation of microtubules in GSK-3β knockdown cells was restored by the expression of a fusion protein of BICD1 and DIC. Therefore, GSK-3β could play a role in stabilizing the formation of a complex between dynein and BICD, resulting in anchoring microtubules to the centrosome.

As Mishappen, a fly homologue of the vertebrate Nck-interacting kinase (NIK) (Houalla *et al*, 2005), and Nek8 (Holland *et al*, 2002) are known to phosphorylate BICD, it is possible that multiple kinases are involved in the BICD phosphorylation. However, in our hands, mammalian NIK did not phosphorylate mammalian BICD1 (data not shown). Although Nek8 is known to localize to the Golgi apparatus or primary cilia but not at the centrosome, knockdown of Nek8 does not affect the ciliogenesis (Mahjoub *et al*, 2005), indicating that a role of Nek8 in the microtubule organization is not clear.

Molecular mechanism by which GSK-3β and BICD regulate microtubule anchoring to centrosome

The localization and function of proteins involved in the microtubule anchoring, such as ninein, PCM-1, and centrin 3, are regulated by dynein–dynactin-mediated minus end-directed transport (Dammermann and Merdes, 2002). We showed that knockdown of GSK-3β or BICD1/2 does not affect the localization of γ-tubulin and centrin 3. These results are consistent with the microtubule regrowth assay in which microtubule aster appeared shortly after regrowth in GSK-3β or BICD1/2 knockdown cells. Therefore, GSK-3β and BICD are not involved in recruitment of γ-TuRC to the centrosome or the regulation of the γ-TuRC activity at the centrosome. As the centrosomal localization of PCM-1 was not affected in BICD1/2 knockdown cells but it was disrupted in GSK-3β knockdown cells, GSK-3β may determine the localization of PCM-1 in cooperation with protein(s) other than BICD.

Ninein was not observed in the centrosome in GSK-3β or BICD1/2 knockdown cells, indicating that GSK-3β and BICD are involved in the centrosomal localization of ninein. Ninein is well known as a minus-end-capping protein and a component of anchoring complex, and tethers microtubules to the centrosome (Bornens, 2002; Delgehyr *et al*, 2005). We found that BICD associates with ninein at the minus end of taxol-induced microtubule bundle. Furthermore, BICD and ninein formed a complex in a biochemical immunoprecipitation assay. Therefore, these results suggest that ninein is one of the cargos of BICD-containing dynein–dynactin complex and that GSK-3 regulates the binding of BICD and dynein, thereby playing a role in transporting ninein to the centrosome. It has been reported that a recessive mutant of the fly homologue of BICD, BICD, disrupts the formation and maintenance of the polarized microtubules, which are essential for the differentiation of *Drosophila* oocytes (Theurkauf *et al*, 1993), indicating that BICD would regulate microtubule focusing at the centrosome beyond the species. The involvement of Shaggy in the functions of BICD remains to be elucidated.

At present, we cannot exclude the possibility that GSK-3β and BICD have a function in stabilization of microtubules and that they are involved in the microtubule anchoring itself. However, this former possibility is unlikely as it has been reported that centrosome-free microtubules in epithelial cells are stable (Rodionov *et al*, 1999), and indeed we observed clear microtubules, although they were not focused to the centrosome, in GSK-3β knockdown cells. Therefore, it is conceivable that GSK-3β and BICD can be involved in the transport of proteins such as ninein and anchoring function.

Materials and methods

Immunocytochemistry

The immunocytochemical analyses of the cultured cells were performed as described (Hino *et al*, 2003; Yamamoto *et al*, 2003) except that the cultured cells were fixed with 100% methanol for 10 min at −20°C or were simultaneously fixed and permeabilized with phosphate buffered saline (PBS) containing 3.7% paraformaldehyde and 0.05% Triton X-100.

For quantitative measurement of focused microtubules, background of all images was subtracted and the integrated intensity of microtubules around the centrosome was divided by the average of three points at cell periphery. When the ratio was more than 1.5-fold, cells had clear visible focused microtubule array and were counted as 'cells with focused microtubules'. At least 300 cells were evaluated for each experimental group. All process and measurements were carried out by MetaMorph software (Universal imaging corporation).

Microtubule regrowth assay

The microtubule regrowth assay was carried out as reported previously (Delgehyr *et al*, 2005). Briefly, siRNA-transfected cells were treated with 20 μM nocodazole in DMEM for 45 min at 37°C and then nocodazole was removed by washing with PBS and cells were incubated with DMEM at 37°C. After 2–10 min of regrowth, the cells were fixed with PBS containing 3.7% paraformaldehyde and 0.05% Triton X-100, and then stained with anti-β- and γ-tubulin antibodies. Cells with small asters at 2 min and cells with focused

microtubules at 10 min were counted. At least 300 cells were evaluated for each experimental group.

Complex formation and immunoprecipitation

For direct binding of BICD1 to GSK-3β, 0.1 μM His_6-GSK-3β was incubated with 0.5 μM GST-BICD1 or GST in 30 μl of reaction mixture (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, and 1 mM dithiothreitol) for 2 h at 30°C. After GST-BICD1 and GST were precipitated with glutathione-Sepharose, the precipitates were probed with anti-His_6 antibody.

To show the complex formation of BICD1 with DIC, $p150^{glued}$, $p50^{dynamitin}$, or GSK-3 at the endogenous level, HeLa S3 cells (60-mm diameter dish) were lysed in 200 μl of lysis buffer (25 mM Tris–HCl, pH 8, 50 mM NaCl, 0.5% Triton X-100, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 5 mM phenylmethylsulfonyl fluoride). The lysates were immunoprecipitated with anti-BICD1 antibody, and the immunoprecipitates were probed with the indicated antibodies.

To determine which region of BICD1 interacts with GSK-3β, COS cells (60-mm diameter dish) were transfected with various deletion mutants of BICD1 and lysed in 200 μl of lysis buffer. The lysates were immunoprecipitated with anti-Myc antibody, and the immunoprecipitates were probed with anti-Myc and anti-GSK-3β antibodies.

Isolation of centrosome

The centrosome was basically prepared from CHO cells according to the methods described previously (Mitchison and Kirschner, 1986). Five confluent 100-mm dishes of CHO cells were treated with 10 μg/ml of nocodazole (Sigma) and 5 μg/ml of cytochalasin D (Sigma) for 90 min. After the drug treatment, the cells were immediately washed with ice-cold PBS, 0.1 PBS containing 8% sucrose and 8% sucrose in distilled water, and LB (1 mM Tris–HCl at pH 7.5 and 0.1% β-mercaptoethanol). The cells were then lyzed with 1 ml of 0.5% Nonidet P-40 in LB and agitated on ice for 10 min. Then cells were collected and 20 μl of 1/50 volume of 50 × PE (500 mM PIPES/NaOH at pH 7.2, 50 mM EDTA, and 5% β-mercaptoethanol) was added. After the centrifugation for 3 min at 1500 *g* to remove the debris, the supernatants (1 ml) were loaded on a 11-ml of discontinuous sucrose gradients consisting of 40 (3 ml), 50 (3 ml), and 70% (5 ml) (w/w) in gradient buffer (10 mM PIPES/NaOH at pH 7.2, 0.1% Triton X-100, and 0.1% β-mercaptoethanol). The gradient was then centrifuged at 112 000 *g* for 2 h at 4°C in SCP70H2 ultracentrifuge (HITACHI, Tokyo, Japan) using a P40ST swinging rotor. After the ultracentrifugation, the top supernatants were aspirated to the 40% sucrose region and 20 fractions of 0.5 ml each were collected from the top of the gradient. The aliquots were subjected to the SDS–PAGE and probed with indicated antibodies. The centrosome-enriched fraction was spotted onto poly-D-lysin-coated coverslips, fixed and stained with the indicated antibodies.

Others

Yeast two-hybrid screening and *in vitro* phosphorylation of BICD1 by GSK-3β were carried out as described previously (Ikeda *et al*, 1998).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

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