A novel Crumbs3 isoform regulates cell division and ciliogenesis via importin β interactions

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The Crumbs family of apical transmembrane proteins regulates apicobasal polarity via protein interactions with a conserved C-terminal sequence, ERLI. However, one of the mammalian Crumbs proteins, Crumbs3 (CRB3) has an alternate splice form with a novel C-terminal sequence ending in CLPI (CRB3-CLPI). We report that CRB3-CLPI localizes to the cilia membrane and a membrane compartment at the mitotic spindle poles. Knockdown of CRB3-CLPI leads to both a loss of cilia and a multinuclear phenotype associated with centrosomal and spindle abnormalities. Using protein purification, we find that CRB3-CLPI interacts with importin β-1 in a Ran-regulated fashion. Importin β-1 colocalizes with CRB3-CLPI during mitosis, and a dominant-negative form of importin β-1 closely phenocopies CRB3-CLPI knockdown. Knockdown of importin β-1 blocks targeting of CRB3-CLPI to the spindle poles. Our data suggest an expanded role for Crumbs proteins in polarized membrane targeting and cell division via unique interactions with importin proteins.

Introduction

For epithelial cells to function, they must polarize into apical and basolateral membranes. The basolateral membrane is defined by lateral cell–cell adhesion and basal interactions with the extracellular matrix. In mammalian epithelia, the tight junction acts as a landmark separating the apical from the basolateral surface. Work over the last 20 yr has begun to elucidate the molecular mechanisms that contribute to the formation of apicobasal polarity in epithelial cells (Nelson, 2003). Important to this progress were studies in *Drosophila melanogaster* and *Caenorhabditis elegans* that elucidated key proteins that were necessary for cell polarization (Knust and Bossinger, 2002; Schneider and Bowerman, 2003). These included a scaffold protein called Stardust and an apical transmembrane protein called Crumbs. Crumbs is a protein that defines the apical membrane, as overexpression leads to an expansion of the apical membrane in *D. melanogaster* epithelia (Wodarz et al., 1995). In mammals, there are three isoforms of Crumbs. Crumbs1 was first identified as RP12, a gene mutated in a subset of patients with retinitis pigmentosa and Leber congenital amaurosis (den Hollander et al., 1999). Crumbs2 is found in the brain, eye, and kidney, but its function is unclear (van den Hurk et al., 2005).

We and others have extensively characterized Crumbs3 (CRB3; Makarova et al., 2003; Roh et al., 2003; Lemmers et al., 2004). The expression of CRB3 is much broader than the other mammalian Crumbs isoforms. CRB3 has been shown to be important for epithelial polarity and tight junction formation. Recently, we have also shown an important role for CRB3 in ciliogenesis by epithelial cells, and similar results have been obtained in zebrafish (Fan et al., 2004; Omori and Malicki, 2006). Although CRB1 and CRB2 isoforms as well as *D. melanogaster* Crumbs have a large extracellular domain with EGF and Laminin repeats, CRB3 has only a small extracellular domain. However, all Crumbs proteins have a highly conserved intracellular domain that ends with the sequence ERLI. This sequence binds to at least two scaffold proteins that are important for cell polarization, Stardust/PALS1 (protein associated with Lin-7) and Par6 (Bachmann et al., 2001; Hong et al., 2001; Roh et al., 2002; Lemmers et al., 2004). The binding of Crumbs to these scaffold proteins is crucial for it to act as an effector in polarity determination and tight junction formation (Klebes and Knust, 2000; Roh et al., 2003; Fogg et al., 2005). However, it has been clear since the identification of mammalian CRB3 that there is an alternate splice form that ends with the C-terminal sequence, CLPI. In this paper, we describe an important role for this CRB3 isoform in ciliogenesis as well as cell division and report its interaction with importin β-1

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Abbreviations used in this paper: EST, expressed sequence tag; shRNA, short hairpin RNA; wt, wild-type.

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Results

CRB3-CLPI is a splice form of the polarity protein Crumbs3 and localizes in primary cilia

A splice form of CRB3 (CRB3 isoform b) is generated by alternate splicing within the fourth exon of the CRB3 gene, leading to a divergent 23-amino-acid sequence at its C terminus ending in the sequence CLPI (Fig. 1 a). Inspection of expressed sequence tags (ESTs) indicate that the CRB3-CLPI splice form can be found in human, mouse, rat, cow, and dog, but apparently not in *D. melanogaster* or zebrafish. EST prevalence indicates a wide tissue expression for both CRB3-ERLI and CRB3-CLPI in rodent and human.

We generated and purified polyclonal antibodies against the last 20 amino acids of CRB3-CLPI. Using immunoblotting, we detected expression of CRB3-CLPI in multiple cell lines (Fig. 1 b). As we had previously found with CRB3-ERLI (Makarova et al., 2003), multiple forms of CRB3-CLPI were seen on blotting, which is due, at least in part, to differential glycosylation. Our previous work indicated that CRB3-ERLI is localized to the apical surface, tight junction, and cilia in MDCK cells (Makarova et al., 2003; Fan et al., 2004). We were not able to detect specific localization of CRB3-CLPI in newly polarized MDCK cells that did not have cilia. However, once the cells fully differentiated, we could detect endogenous CRB3-CLPI in cilia (Fig. 1 c, top). To confirm this result, we expressed a full-length Myc–CRB3-CLPI construct in MDCK cells. In this construct, the Myc tag was placed in the extracellular domain near the signal peptide. We detected this transfected Myc–CRB3-CLPI using two methods. One used CRB3-CLPI antibody at a high dilution of 1:1,000 that could not detect endogenous CRB3-CLPI (Fig. 1 c, middle); the other used anti-Myc 9E10 monoclonal antibody (Fig. 1 c, bottom). With both these reagents, we could detect cilia staining of the transfected CRB3-CLPI. We further verified the specificity of the CRB3-CLPI antibody by adding the CRB3-CLPI antigenic peptide to the immunostaining. This peptide blocked the anti–CRB3-CLPI staining of both endogenous and transfected proteins (Fig. S1 a, available at http://www.jcb.org/cgi/content/full/jcb.200609096/DC1).

CRB3-CLPI localizes to the spindle poles during cell division

Cilia have been the focus of recent studies because of the association of cilia-localized proteins with many human diseases, including polycystic kidney disease (Badano et al., 2005; Hildebrandt and Otto, 2005). It has been suggested that defects in spindle pole polarity contribute to the genesis of polycystic kidney disease (Germino, 2005; Fischer et al., 2006). Accordingly, we studied the localization of CRB3-CLPI during the cell cycle. As we previously stated, we could not detect the localization of endogenous CRB3-CLPI during interphase in MDCK cells.

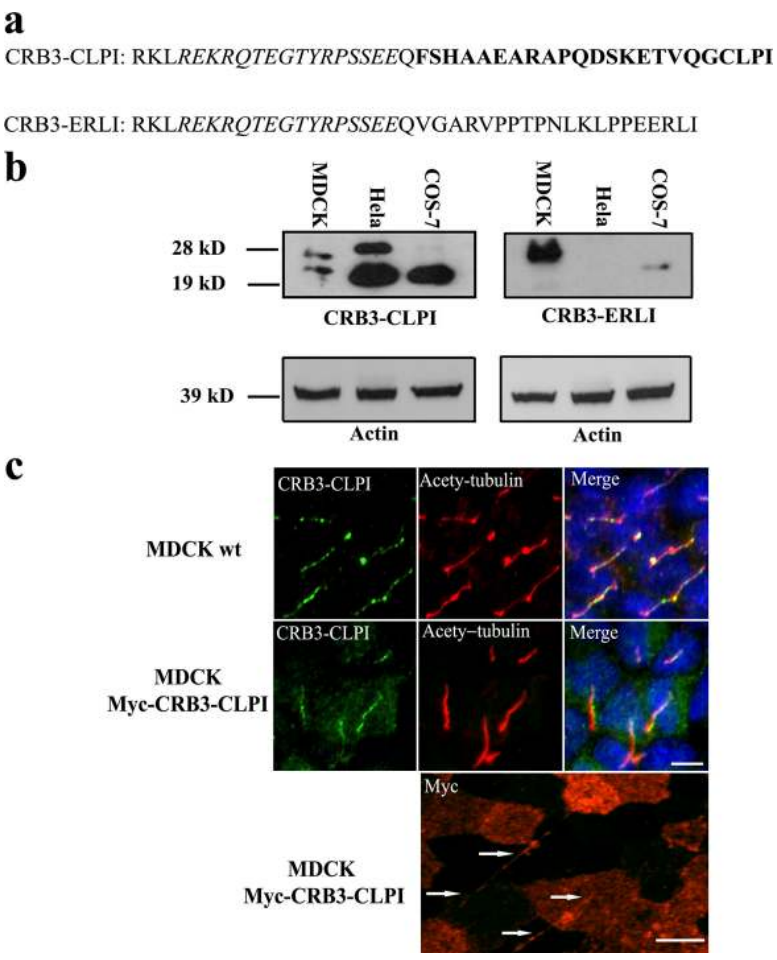

Figure 1. **CRB3-CLPI is a splice form of the polarity protein Crumbs3 and localizes in primary cilia.** (a) Sequence comparison of the intracellular domains of human CRB3-CLPI and CRB3-ERLI. These two proteins have an identical extracellular domain, transmembrane domain, and 4.1 binding domain (italics). The last 23 amino acids of CRB3-CLPI are distinct from CRB3-ERLI (bold). (b) CRB3-CLPI blotting in different cell lines. Lysates from MDCK, HeLa, and COS-7 cells were separated by Bis-Tris gel and followed by blotting with rabbit anti–CRB3-CLPI and –CRB3-ERLI antibodies. Anti-actin blot serves as a loading control. (c) CRB3-CLPI localizes to cilia. (top) Staining with CRB3-CLPI (green) shows endogenous protein in the cilia: acetylated tubulin (cilia marker in red) in MDCK. (middle and bottom) Myc–CRB3-CLPI also shows cilia localization in MDCK cells. Middle panel shows staining with dilute anti–CRB3-CLPI at 1:1,000 that does not detect endogenous CRB3-CLPI and mouse anti–acetylated tubulin (red). Bottom panel shows staining with mouse anti-Myc (red; arrows indicate the cilia staining). Bars, 5 μm.

However, at prophase, CRB3-CLPI was concentrated around the centrosomes (Fig. 2 a). As the cells progressed through metaphase and anaphase, there was a close colocalization of CRB3-CLPI and centrosomes. This continued through anaphase, but at telophase, CRB3-CLPI appeared diffuse throughout the cell and could not be clearly localized.

We also tested the localization of the Myc–CRB3-CLPI protein during the cell cycle staining both with the diluted 1:1,000 CRB3-CLPI antibody or Myc antibody (Fig. 2, b and c). A sharp localization of the transfected CRB3-CLPI protein to a pericentrosomal location in metaphase could be detected using either antibody. In some of these cells, CRB3-CLPI was also detected at the cell cortex, but this was felt to be due to overexpression, as this was never seen when staining the endogenous protein. This result indicates that CRB3-CLPI marks a pericentrosomal membrane component, as the Myc tag is in the

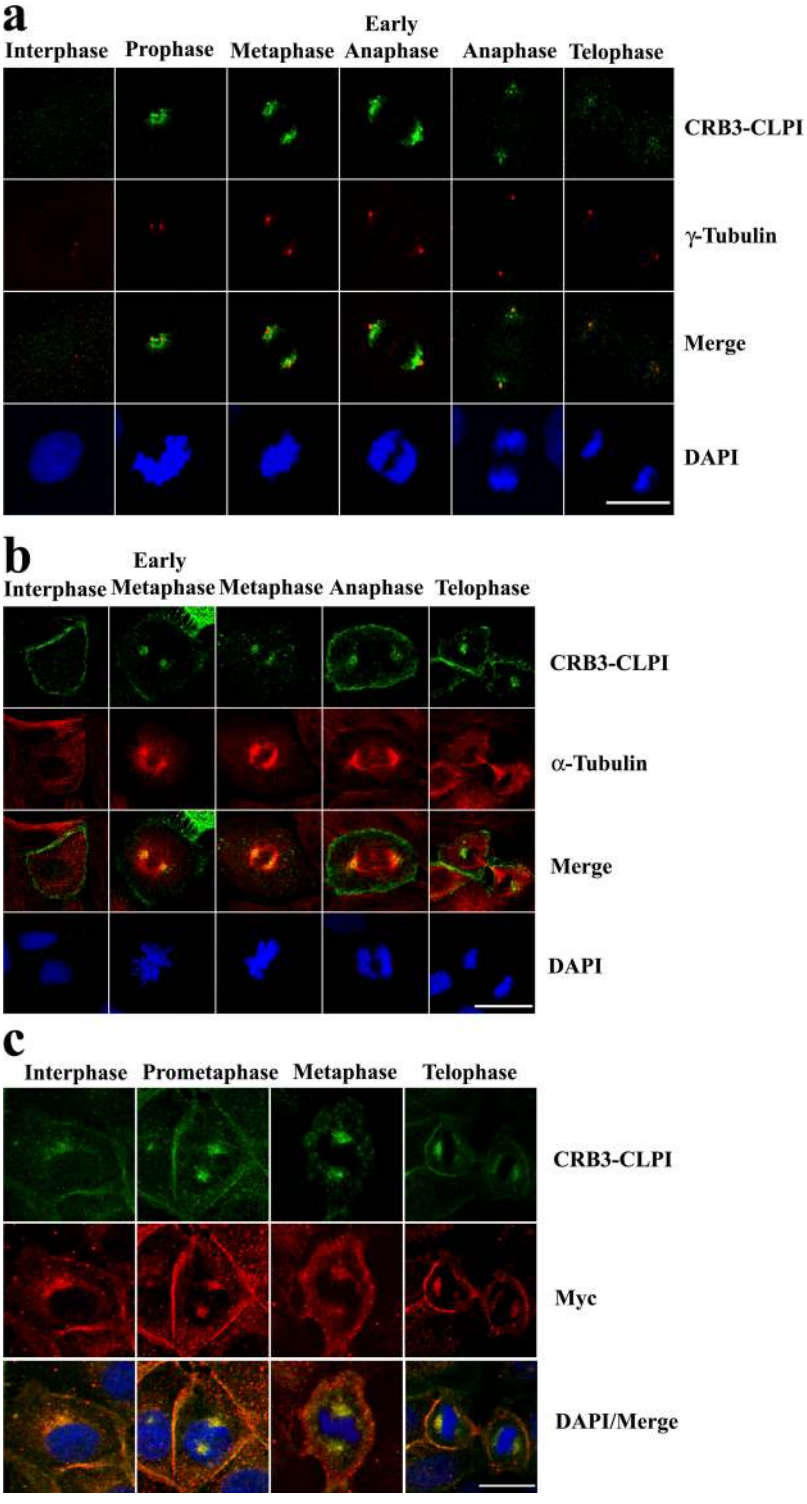

Figure 2. **CRB3-CLPI localizes to the spindle poles during cell division.** (a) Immunostaining of MDCK cells with anti–CRB3-CLPI (green) and mouse anti–γ-tubulin (red) during the cell cycle. DAPI indicates nuclear staining (blue). (b) MDCK cells stably expressing Myc–CRB3-CLPI were immunostained using dilute rabbit anti–CRB3-CLPI (green; 1:1,000) and mouse anti–α-tubulin (red). Staining untransfected cells using CRB3-CLPI antibody at 1:1,000 concentration yields no staining (not depicted). (c) Same cells as in panel b stained with diluted CRB3-CLPI (green) and mouse anti-Myc (red). Bars, 5 μm.

extracellular domain and the CRB3-CLPI antibody epitope is in the intracellular domain. We obtained similar CRB3-CLPI staining results in Cos-7 cells (Fig. S1 b). Cos-7 cells express more endogenous CRB3-CLPI than MDCK cells, and during interphase, the CRB3-CLPI could also be detected in a pericentrosomal region, consistent with Golgi localization. During mitosis, the CRB3-CLPI in Cos-7 cells localized to a tight dot surrounding the centrosome, as was seen with MDCK cells. Interestingly, we also found that CRB3-CLPI localized to the midbody during cytokinesis (Fig. S1 b). The localization of the CRB3-CLPI was markedly different from that seen with CRB3-ERLI. At interphase in MDCK cells, CRB3-ERLI was seen apically and at tight junctions as previously described (Fig. S1 c). During metaphase, anaphase, and telophase, however, CRB3-ERLI was diffuse throughout the MDCK cells. There was no localization around the spindle poles, as was seen with CRB3-CLPI.

CRB3-CLPI knockdown leads to multinuclei, spindle, cilia, and centrosome abnormalities

Our previous studies, as well as studies in zebrafish, have demonstrated that removal of Crumbs affects ciliogenesis (Fan et al., 2004; Omori and Malicki, 2006). However, in our previous studies, we used short hairpin RNA (shRNA) constructs that could have eliminated both the CRB3-ERLI and CRB3-CLPI isoforms. Accordingly, we transfected MDCK cells with a pSilencer shRNA specifically directed toward CRB3-CLPI or CRB3-ERLI and selected stable cell lines. We were able to obtain a considerable knockdown of the CRB3-CLPI protein (Fig. 3 a). In CRB3-CLPI, no defects in tight junctions were detected, unlike what was seen with CRB3-ERLI–specific knockdowns (Fig. 3 b). However, we noted that many cells with CRB3-CLPI knockdown displayed a multinuclear phenotype that was not seen in the CRB3-ERLI knockdown (Fig. 3, c and d). This was seen in both clones 1 and 2 MDCK knockdown cell lines, which were generated using different shRNA constructs (see Materials and methods). Because the expression of CRB3-ERLI was slightly increased (Fig. 3 a, middle) in cells with CRB3-CLPI knockdown, we needed to exclude the possibility that the multinuclear phenotype of CRB3-CLPI knockdown was a consequence of the increased level of CRB3-ERLI expression. We studied stable MDCK cell lines that overexpress CRB3-ERLI (Roh et al., 2003). However, the multinuclear phenotype was not detected in these cells, indicating that the overexpression of CRB3-ERLI did not contribute to this phenotype (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200609096/DC1). This multinuclear phenotype was associated with markedly abnormal mitotic spindles (Fig. 4, a and b). The most common defect seen was multiple spindle poles (~80%), but misaligned and disorganized bipolar spindles were also seen.

CRB3-CLPI knockdown cells often contained supernumerary centrosomes (Fig. 4 c), and this likely contributed to these cells having multiple spindle poles. We found that the presence of supernumerary centrosomes coincided with multinuclei in CRB3-CLPI knockdown cells (Fig. 4 c). Almost all the multinucleated cells had more than two centrioles (supernumerary centrosomes), whereas only 12% of mononuclear cells had more than two centrioles (Fig. 4 d). We found that once the CRB3-CLPI knockdown cells grew to confluence, the multinuclear cells with supernumerary centrosomes were markedly

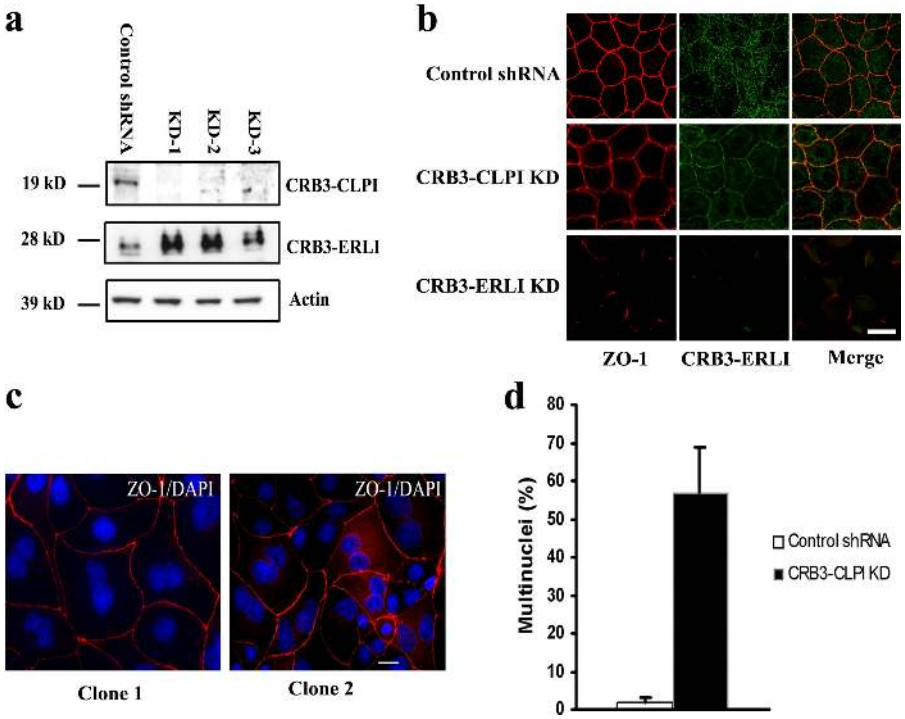

Figure 3. **CRB3-CLPI knockdown leads to multinuclear cells.** (a) Western blot analysis was used to determine the level of CRB3-CLPI in MDCK cells stably expressing shRNA against CRB3-CLPI (clones KD-1 from CRB3 shRNA targeting sequence 1 and KD-2, KD-3 from CRB3 shRNA targeting sequence 2; see Materials and methods) and control shRNA (top). Blotting is also shown for CRB3-ERLI (middle) and an actin loading control (bottom). (b) CRB3-ERLI knockdown (KD) but not CRB3-CLPI knockdown induces tight junction defects in MDCK cells. CRB3-ERLI knockdown (bottom), CRB3-CLPI knockdown (middle), and control shRNA (top) stable cell lines were grown on filters to confluence. 6 h after calcium switch, cells were fixed and stained with tight junction marker: mouse anti–ZO-1 (red) and rabbit anti–CRB3-ERLI (green). (c) CRB3-CLPI knockdown induces a multinuclear phenotype in MDCK cells. MDCK CRB3-CLPI knockdown stable cell line clone 1 and clone 2 (shRNA targeting sequence 1 and 2, respectively) were stained with mouse anti–ZO-1 (red) to indicate the cell boundaries; DAPI (blue) indicates nuclei. (d) Percentage of cells with multinuclei was calculated from CRB3-CLPI knockdown and control shRNA. 100 cells each from three CRB3-CLPI knockdown clones and control shRNA clones were evaluated. Results are the mean of three individual clones, shown as mean ± SD. Bars, 5 μm.

reduced and mononuclear cells predominated. This was not due to loss of the CRB3-CLPI knockdown, as when these cells were diluted and replated, the multinuclear cells reappeared (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200609096/DC1). We hypothesize that as cells reached confluence, the multinuclear cells disappeared, possibly as a result of apoptosis (Meraldi et al., 2002; Shi and King, 2005; Srsen and Merdes, 2006). The cell division defect appears to be stochastic, as a certain fraction of the cells are mononuclear, divide properly, and predominate in the confluent monolayer. We next examined the role of CRB3-CLPI in ciliogenesis. We allowed cells to grow on filters for 7 d to achieve confluence to the point where multinuclear cells were rare (Fig. S3). At this stage, we found that CRB3-CLPI knockdown MDCK cells had a defect in ciliogenesis (Fig. 4 e). The loss of cilia could be due, in part, to a polarity defect, as many knockdown cells failed to localize centrosomes and Golgi during polarization to a subapical localization (Fig. 4 f).

To determine if these phenotypes were specific for CRB3-CLPI knockdown cells, we reexpressed Myc–CRB3-CLPI in these knockdown cells using a cDNA that was resistant to the shRNA. We were able to demonstrate that reexpression of the Myc–CRB3-CLPI but not vector alone could reverse the multinuclear phenotype (Fig. 5, a and b). In addition, we noted that the abnormal localization of centrosomes in growth-arrested CRB3-CLPI knockdown cells could be reversed (Fig. 5 c). Reexpression of CRB3-CLPI not only reversed the abnormal centrosomal phenotype but also restored Golgi localization to the apical region of the MDCK cells. However, we were still not able to detect cilia in these rescued cells, perhaps because of the level of overexpression of Myc–CRB3-CLPI or as an effect of the Myc tag.

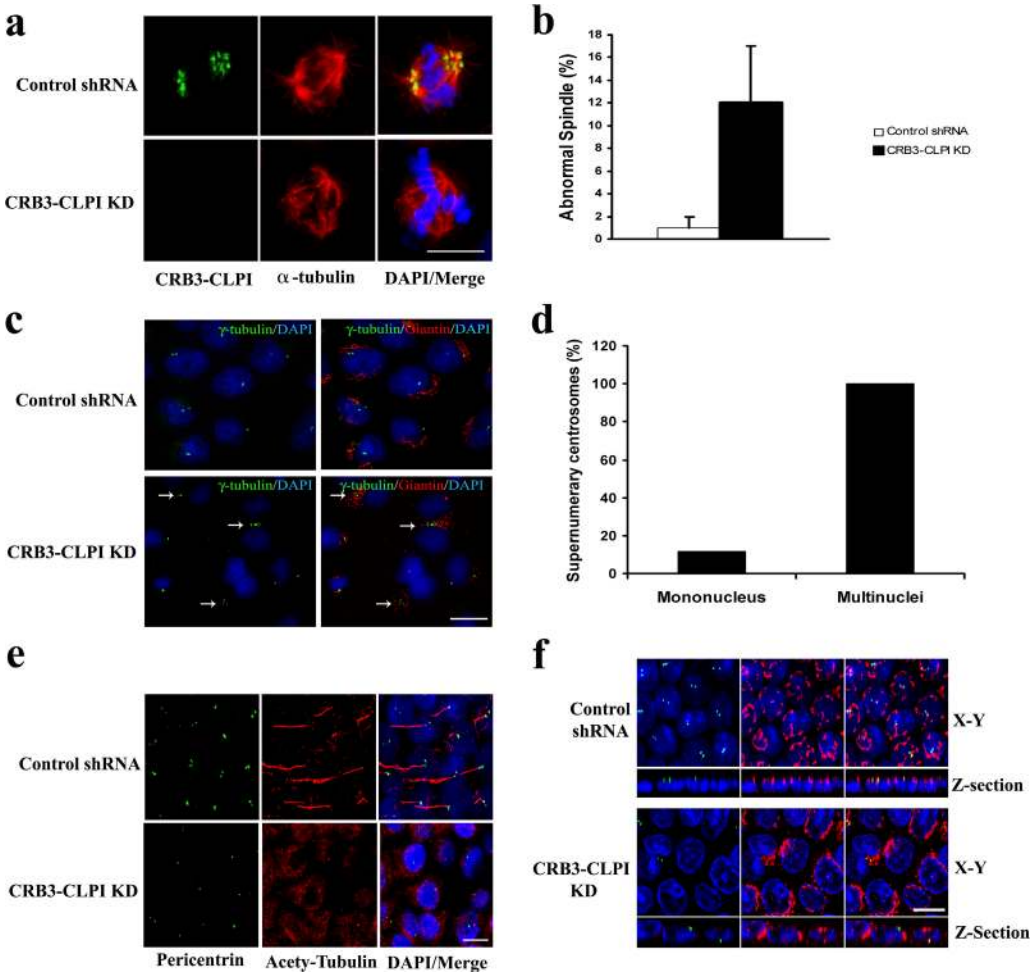

Figure 4. **CRB3-CLPI knockdown leads to spindle, cilia, and centrosome abnormalities.** (a) CRB3-CLPI knockdown (KD) results in abnormal mitotic spindles in MDCK cells. Mitotic cells were stained with anti–CRB3-CLPI (green) and anti–α tubulin (red). DAPI (blue) shows the nuclei. (b) Percentage of cells with abnormal spindles in three CRB3-CLPI knockdown and control shRNA clones. Results are the mean of three individual clones, shown as mean ± SD. $n = 100$. (c) CRB3-CLPI knockdown results in supernumerary centrosomes in MDCK cells. CRB3-CLPI knockdown (bottom) and control shRNA (top) cells were grown on chamber slides for 1 d and stained with anti-Giantin (red) and anti–γ-tubulin (green) to show the Golgi apparatus and centrosome. Arrows point to cells with supernumerary centrosomes. (d) Percentage of cells with CRB3-CLPI knockdown and supernumerary centrosomes in mononuclear and multinuclear cells. Results are the mean of three individual clones. $n = 100$. (e) CRB3-CLPI knockdown is associated with a cilia defect. CRB3-CLPI knockdown MDCK stable cells and control shRNA cells were grown on filters for 7 d. Staining was then performed with anti–acetylated tubulin (red) and rabbit anti-pericentrin (green). (f) Abnormal centrosome and Golgi orientation in CRB3-CLPI knockdown MDCK cells. CRB3-CLPI knockdown and control shRNA cells were grown on filters for 7 d and then stained with the Golgi marker anti-GM130 (red) and pericentrin (green). DAPI (blue) shows the nuclei. Bars, 5 μm.

CRB3-CLPI interacts and colocalizes with importin β-1

The CRB3-ERLI protein can interact with the polarity proteins PAR6 and PALS1 (Roh et al., 2002; Lemmers et al., 2004). However, we were not able to demonstrate such interactions with CRB3-CLPI (unpublished data). Accordingly, we performed large-scale anti-Myc immunoprecipitations from MDCK cells expressing Myc–CRB3-CLPI, Myc–CRB3-ERLI, or vector alone and looked for differences in interacting proteins. A specific band of ∼100 kD detected by Myc–CRB3-CLPI immunoprecipitation was excised and sent for liquid chromatography/mass spectrometry (MS; Fig. 6 a). Analysis yielded 19 matching peptides and 28% coverage for mouse importin β-1. MS/MS analysis yielded two peptides that matched mouse importin β-1, AAVENLPTFLVELSR and WLAIDANAR.

Importin β-1 directly or via its interactions with importin α isoforms and Ran GTPase facilitates trafficking of proteins to the nucleus (Macara, 2001; Bednenko et al., 2003). However, recent studies have also suggested an important role for these proteins in mitotic spindle generation and centrosome maintenance (for review see Harel and Forbes, 2004); thus, it appeared that importin β-1 was a good candidate for a CRB3-CLPI binding partner. Indeed, we were able to show that importin β-1 colocalized with CRB3-CLPI during mitosis (Fig. 6 b). This was in agreement with previous reports on importin β-1 targeting spindle assembly factors during mitosis (Nachury et al., 2001; Wiese et al., 2001; Ciciarello et al., 2004). In addition, we were able to show that importin β-1 colocalized to the cilia with CRB3-CLPI (Fig. 6 c). We next examined the coimmunoprecipitation of CRB3-CLPI with importin β-1. Myc–CRB3-CLPI, Myc–CRB3-ERLI, or MDCK wild-type (wt) cells were transfected and immunoprecipitated with the Myc antibody. We found that endogenous importin β-1 would coimmunoprecipitate with Myc–CRB3-CLPI but not with Myc–CRB3-ERLI or in control MDCK cells (Fig. 6 d). This result was confirmed by coexpressing Flag–importin β-1

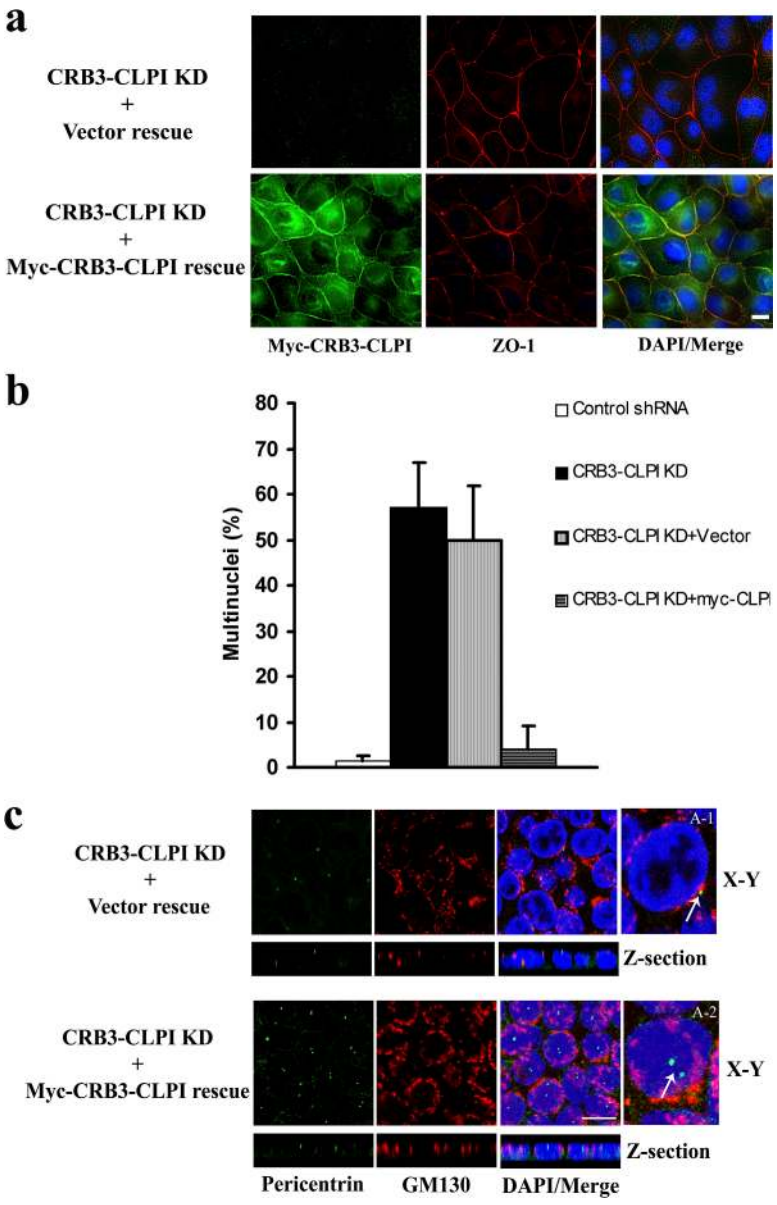

Figure 5. **Exogenous Myc–CRB3-CLPI is able to rescue the multinuclear and centrosome defect caused by CRB3-CLPI knockdown (KD).** (a) pcDNA 3.1 Myc–CRB3-CLPI and vector alone transfected MDCK CRB3-CLPI knockdown stable cell lines were stained with diluted rabbit anti–CRB3-CLPI (1:1,000; green) and mouse anti–ZO-1 (red) antibodies. DAPI indicates nuclei. (b) Percentage of cells with multinuclei from control shRNA, CRB3-CLPI shRNA, CRB3-CLPI shRNA$^+$ vector rescue, and CRB3CLPI shRNA$^+$ Myc–CRB3-CLPI rescue. Results are the mean of three individual clones, shown as mean ± SD. n = 100. (c) Exogenous Myc–CRB3-CLPI can rescue the centrosome and Golgi polarization defects induced by CRB3-CLPI knockdown. MDCK CRB3-CLPI knockdown cells stably expressing exogenous Myc–CRB3-CLPI or vector alone were grown on filters for 7 d. Immunostaining was then performed with anti-GM130 (red) and pericentrin (green). DAPI (blue) shows the nuclei. A-1 and A-2 are enlargements of GM130 and pericentrin staining from vector alone and exogenous Myc–CRB3-CLPI rescue. Arrows indicate centrosomes. Bars, 5 μm.

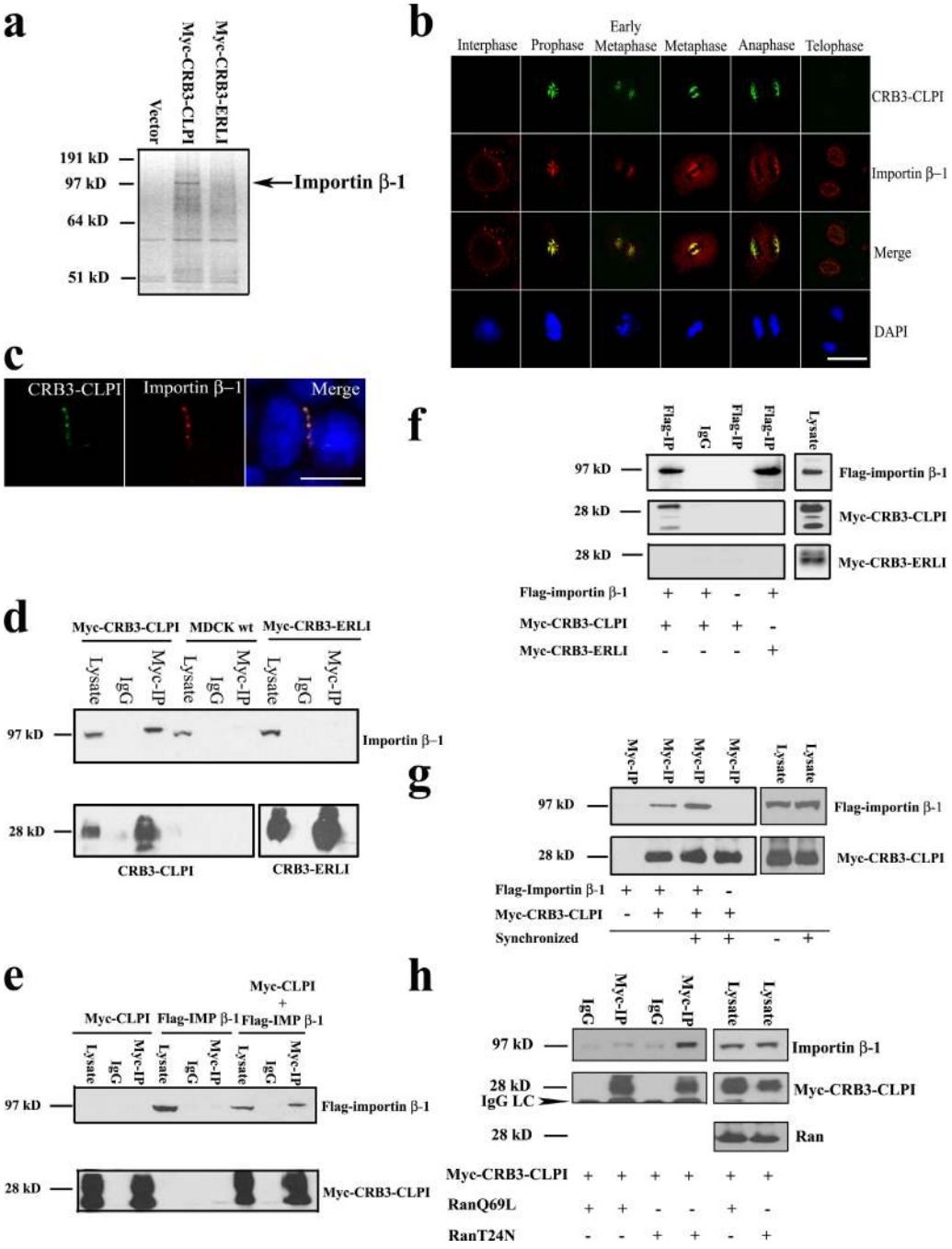

Figure 6. **CRB3-CLPI interacts and colocalizes with importin β-1.** (a) Myc–CRB3-CLPI coimmunoprecipitates a 97-kD protein. Large-scale anti-Myc immunoprecipitation from MDCK cell lines stably expressing Myc–CRB3-CLPI, Myc–CRB3-ERLI, or vector alone were visualized by silver staining. Arrow points to a 97-kD protein (importin β-1) that interacts with Myc–CRB3-CLPI but not with Myc–CRB3-ERLI or vector alone. (b) Importin β-1 colocalizes with CRB3-CLPI during mitosis. MDCK wt cells were stained with rabbit anti–CRB3-CLPI (green) and mouse anti–importin β-1 (red) antibodies. DAPI indicates nuclear staining. (c) Importin β-1 colocalizes with CRB3-CLPI in cilia. MDCK wt cells were grown on filters for 7 d to allow cilia growth. Cells were then stained as in panel a. (d) Myc–CRB3-CLPI coimmunoprecipitates with endogenous importin β-1 but not Myc–CRB3-ERLI in MDCK cells. Myc–CRB3-CLPI or Myc–CRB3-ERLI MDCK stable cell lines or untransfected MDCK wt cells were lysed with Triton X-100 lysis buffer and immunoprecipitated with anti-Myc antibody (Myc-IP) and subsequently immunoblotted for importin β-1, CRB3-CLPI, and CRB3-ERLI. Mouse IgG serves as an immunoprecipitation control. (e) Myc–CRB3-CLPI precipitates Flag–importin β-1. COS-7 cells were transiently transfected with Myc–CRB3-CLPI (Myc-CLPI), Flag–importin β-1 (Flag-IMP β-1), or both. Lysates from these cells were then subjected to anti-Myc immunoprecipitation and immunoblotted with anti-Myc or anti-Flag. (f) Flag–importin β-1 precipitates Myc–CRB3-CLPI but not Myc–CRB3-ERLI. Cos-7 cells were transiently transfected with Myc–CRB3-CLPI or Myc–CRB3-ERLI with or without Flag–importin β-1. Lysates were then subjected to immunoprecipitation with mouse IgG or anti-Flag antibody and blotted with anti-Flag and anti–CRB3-CLPI or anti–CRB3-ERLI. (g) Enhanced CRB3-CLPI–importin β-1 interactions in synchronized cells. HeLa cells were transfected with combinations of Myc–CRB3-CLPI and Flag–importin β-1 and synchronized using nocodazole as described in Materials and methods. 30 min after removing nocodazole, cells were lysed, immunoprecipitated, and blotted as previously described. More than 90% of cells entered mitosis after release of the mitotic block. (h) RanQ69L, but not RanT24N, disrupts CRB3-CLPI interactions with importin β-1. RanQ69L or RanT24N (6 μg) were cotransfected with 2 μg Myc–CRB3-CLPI into Cos-7 cells, and 24 h later, cells were lysed and anti-Myc immunoprecipitation was performed. Immunoprecipitates and lysates were then separated by Bis-Tris gel and blotted with anti–importin β-1, anti-Myc, and anti-Ran. Arrow indicates the antibody's light chain (IgG LC). Lysate lanes represent 10% input of total lysate used for immunoprecipitation in each experiment in this figure. Bars, 5 μm.

and Myc–CRB3-CLPI in Cos-7 cells. We were able to immunoprecipitate Flag–importin β-1 with anti-Myc antibodies only in cells expressing both tagged proteins (Fig. 6 e). Similarly, Flag immunoprecipitation brought down Myc–CRB3-CLPI but not Myc–CRB3-ERLI (Fig. 6 f). Similarly, full-length importin β-1 GST fusion protein was also able to precipitate Myc–CRB3-CLPI from lysates (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200609096/DC1). The ability of the importin β-1 GST fusion protein to bind importin α is used as a control in Fig. S4. We noted that the colocalization of Myc–CRB3 CLPI and importin β-1 was strongest during cell division and found that Myc–CRB3-CLPI more strongly interacted with importin β-1 shortly after release of a mitotic block (Fig. 6 g).

Importin β-1 often interacts with cargo via importin α, and this interaction is regulated by the Ran small GTPase. However, we were not able to detect importin α in the CRB3-CLPI immunoprecipitates (unpublished data). This may not be surprising, as it has been demonstrated that importin β-1 can bind cargo proteins in the absence of importin α (Chook and Blobel, 2001; Blower et al., 2005). However, we were able to demonstrate that Ran regulates the interaction of importin β-1 with CRB3-CLPI (Fig. 6 h). Transfection of GTP-Ran (Q69L) but not GDP-Ran (T24N) blocked the interaction of CRB3-CLPI with importin β-1.

To further assess the functional importance of the interaction, we generated a dominant-negative importin β-1 missing the N terminus and transfected it into MDCK cells. This dominant-negative form of importin β-1 is missing the Ran GTPase binding motif but is still able to interact with Myc–CRB3-CLPI (Fig. 7 a) and target to spindle poles (Fig. S5 a, available at http://www.jcb.org/cgi/content/full/jcb.200609096/DC1). We were able to show that overexpression of this dominant-negative importin β-1 closely phenocopied CRB3-CLPI shRNA with multinuclear cells (Fig. 7, b and c), and abnormal spindle poles (Fig. 7, d and e) with supernumerary centrosomes (Fig. 7 d). In addition, we also saw the loss of cilia in these cells (Fig. 7 f).

Importin β-1 knockdown induces abnormal mitotic spindles and blocks CRB3-CLPI targeting to spindle poles during mitosis

Finally, we looked at targeting of importin β-1 in cells missing CRB3-CLPI. Although many of these cells showed abnormal spindles with multiple centrosomes, importin β-1 was seen concentrated around the spindle poles, suggesting that the CRB3-CLPI

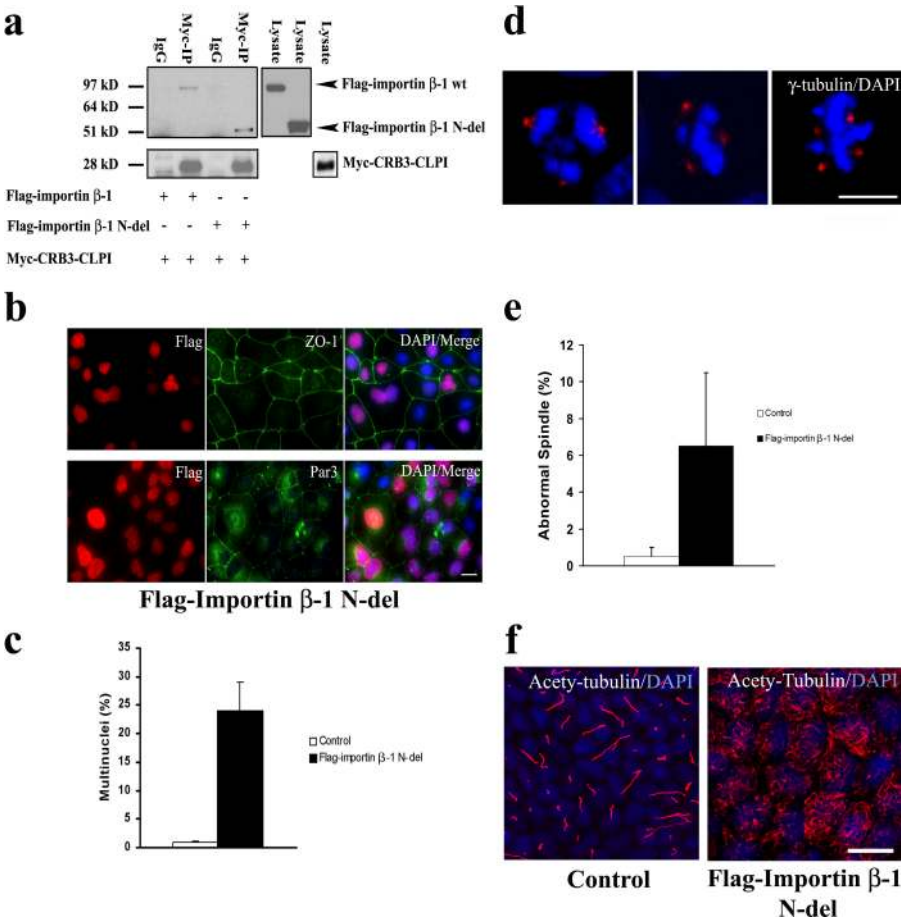

Figure 7. **Stable overexpression of an importin β-1 mutant induces multinuclei, abnormal spindle phenotypes and cilia defects.** (a) Importin β-1 N-deletion (importin β-1 N-del) does not affect the interaction between CRB3-CLPI and importin β-1. Cos-7 cells were transiently transfected with Myc–CRB3-CLPI and Flag–importin β-1 wt or N-del, a dominant-negative form of importin β-1 missing the first 360 amino acids. Lysates were then subjected to immunoprecipitation with mouse IgG or anti-Myc antibody and blotted with anti-Flag or anti-Myc antibodies. Lysate lanes represent 10% of the lysate used for immunoprecipitation. (b) Multinuclei in cells expressing Flag–importin β-1 N-del. MDCK cells expressing Flag–importin β-1 N-del were stained with anti-Flag (red) and anti–ZO-1 or Par3 (green) to demarcate the cell boundary. DAPI stains the nucleus. (c) The percentage of cells with multinuclei was calculated from Flag–importin β-1 N-del and control cells. 100 cells from each clone of Flag–importin β-1 N-del versus control cells were evaluated. Results are the mean of three individual clones, shown as mean ± SD. (d) Multiple spindle poles are present in cells expressing Flag–importin β-1 N-del. Flag–importin β-1 N-del MDCK cells were stained with anti–γ-tubulin (red) and DAPI (blue) to show the centrosome and nuclei. (e) The percentage of cells with abnormal spindles was determined from Flag–importin β-1 N-del and control cells. 100 cells from each clone of Flag–importin β-1 N-del and control cells were evaluated. Results are the mean of three individual clones, shown as mean ± SD. There was a significant difference in wt versus dominant-negative expressing clones ($P < 0.05$; unpaired *t* test). (f) Overexpression of Flag–importin β-1 N-del induces cilia defects. Flag–importin β-1 N-del stable MDCK cells (right) and control cells (left) were grown on filters for 7 d and stained with anti–acetylated tubulin (red) and DAPI (blue). Bars, 5 μm.

was not essential for this targeting of importin β-1 (Fig. 8 a). Next, we examined the effects of importin β-1 knockdown on CRB3-CLPI targeting. We were unable to obtain clonal cells with sustained knockdown of importin β-1, presumably because of toxicity induced by loss of this protein. However, we were able to transiently express double-stranded shRNA and study knockdown cells within 48 h of transfection (Fig. 8 b). As might be expected, cells lacking importin β-1 demonstrated abnormal mitotic spindles (for review see Harel and Forbes, 2004), and these spindles consistently lacked CRB3-CLPI staining (Fig. 8, c and d). This indicates that importin β-1 played an important role in targeting CRB3-CLPI but not vice versa.

Discussion

Studies to date have begun to reveal the role of the Crumbs proteins in multiple developmental systems from *D. melanogaster* to zebrafish. Crumbs proteins have a conserved ERLI motif at their C terminus that binds to PALS1 and Par6 and is crucial for their function (Klebes and Knust, 2000; Lemmers et al., 2004; Fogg et al., 2005). However, there is an alternate splice form of mammalian CRB3 that adds 23 unique amino acids to the C terminus. The CRB3-CLPI isoform concentrates in a membrane compartment that localizes around the centrosome. Loss of CRB3-CLPI leads to defects in spindle assembly, cilia formation, and cell division. CRB3-CLPI interacts in a Ran-regulated fashion with importin β-1, and this interaction appears important for CRB3-CLPI targeting to the pericentrosomal region.

The defect that leads to the multinuclear phenotype most likely represents a cytokinesis defect, and indeed we found CRB3-CLPI localized to the midbody in Cos-7 cells. Studies have pointed to an evolutionarily conserved role for the centrosome in cytokinesis (for review see Baluska et al., 2006). For example, work from Gromley et al. (2003, 2005) described an essential process in which the centrosomal protein Centriolin anchors the exocyst and SNARE complexes and guides vesicle transport to the midbody in the final stages of cytokinesis. Others have shown an important role for the centrosomal Bardet-Biedl syndrome proteins in cytokinesis (Kim et al., 2005).

In addition to a cytokinesis defect, it is also possible that correct localization of this CRB3-CLPI–containing membrane is necessary for mammalian cells to complete cell division, as is seen with members of the Golgi matrix (Sutterlin et al., 2002). Inheritance of Golgi membranes is perhaps the best-studied example of membrane organelle inheritance and is due to vesiculation and dispersion of the membrane (Shorter and Warren, 2002). It has been argued that this diffuse distribution in the cytoplasm of mitotic cells ensures equal inheritance; however, recent studies indicate that mitotic Golgi fragments also align with astral microtubules at the spindle poles in certain cell types (Shorter and Warren, 2002). Indeed, members of the Golgi matrix can regulate cell cycle progression, perhaps ensuring proper Golgi inheritance before cell division can be completed (Sutterlin et al., 2002, 2005). However, the localization of GM130, a membrane Golgi marker, and CRB3-CLPI was not identical during cell division, indicating that CRB3-CLPI marks a different compartment (Fig. S5 b). Early endosomes are another membrane compartment that exists in a pericentrosomal distribution early during cell division and contributes to cytokinesis (Dunster et al., 2002), but we saw no colocalization between early endosome markers and CRB3-CLPI (Fig. S5 b).

In addition to a role in cytokinesis, the CRB3-CLPI–containing pericentrosomal membrane appears to contribute to the formation of the cilia. It has long been known that a pericentrosomal ciliary vesicle covers the centrosome during early ciliogenesis (Sorokin, 1968), and other cilia membrane components localize near the centrosome during cell division (Rundle et al., 2004). We hypothesize that CRB3-CLPI also exists in this early cilia membrane. Loss of cilia was seen with two different shRNAs directed against CRB3-CLPI; however, we could not rescue the cilia defect with shRNA-resistant CRB3-CLPI despite

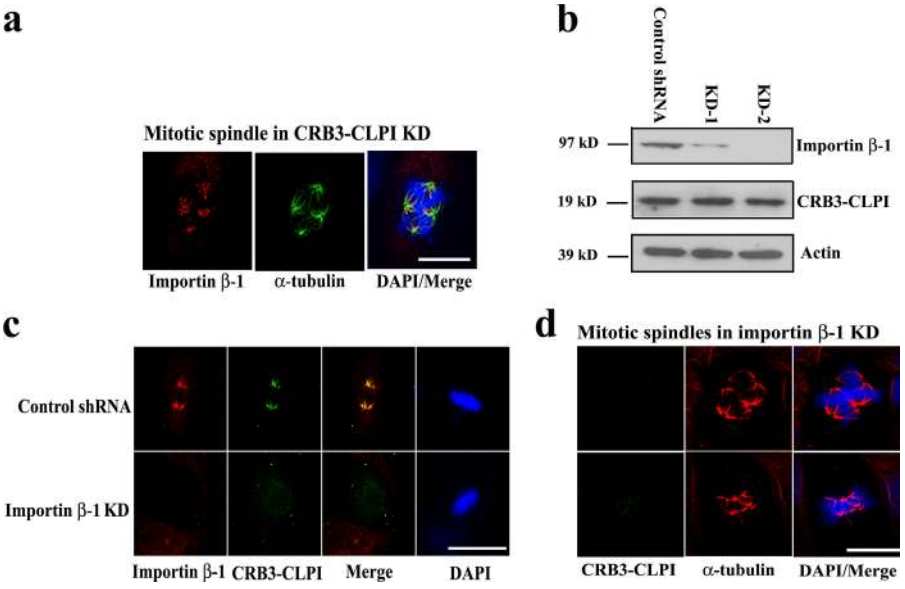

Figure 8. **Importin β-1 knockdown induces abnormal spindles and blocks targeting of CRB3-CLPI to the spindle poles during mitosis.** (a) CRB3-CLPI knockdown (KD) does not affect the importin β-1 spindle pole localization during metaphase. MDCK CRB3-CLPI knockdown cells were grown on chamber slides for 1 d. After fixing, cells were stained with mouse anti–importin β-1 (red) and rat anti–α-tubulin (green). (b) Western blot of importin β-1 in MDCK cells transiently transfected with shRNA against importin β-1 (KD-1 and -2) and control shRNA (top). Endogenous CRB3-CLPI (middle) was not affected, and anti-actin blot (bottom) serves as a loading control. (c) MDCK importin β-1 knockdown (bottom) and control shRNA (top) cells were stained with mouse anti–importin β-1 (red) and rabbit anti–CRB3-CLPI (green) antibodies. (d) Importin β-1 knockdown in mitotic MDCK cells stained with CRB3-CLPI (green) and mouse anti–α-tubulin (red). DAPI indicates the nuclei. Bars, 5 μm.

rescuing the cell division defect. It should be noted that we have not been able to rescue the cilia defect seen in CRB3-ERLI knockdowns either, but the cilia defect with the CRB knockdown has been seen both in mammalian cells and zebrafish (Fan et al., 2004; Omori and Malicki, 2006). Our rescue studies lead to the overexpression of CRB3, and it is well known that overexpression of Crumbs proteins can affect cellular phenotypes (Izaddoost et al., 2002; Pellikka et al., 2002; Roh et al., 2003). A membrane defect is not the only possible mechanism for the lack of cilia in the CRB3-CLPI knockdown cells. We have also seen striking defects in centrosomal and Golgi targeting within the CRB3-CLPI knockdown cells; the exact mechanism of these defects is unclear, as we did not detect interactions of this CRB3 isoform with other polarity proteins, such as PALS1 or Par6. Thus, the exact role of CRB3-CLPI in cilia formation will require additional studies.

Another major finding in our studies is an interaction between CRB3-CLPI and importin β-1. In nuclear translocation, importin β binds cargo directly or indirectly through importin α. Upon entering the nucleus, the cargo is released when importin β binds to the Ran GTPase (Macara, 2001; Nachury et al., 2001; Wiese et al., 2001; Bednenko et al., 2003). CRB3-CLPI may directly bind to importin β-1, as importin α did not immunoprecipitate with CRB3-CLPI. We find that GST–importin β-1 can precipitate CRB3-CLPI from cell lysates (Fig. S4); however, we have not been able to demonstrate that a GST–CRB3-CLPI intracellular domain can precipitate importin β-1 under similar conditions. Thus, it is not yet clear whether the interaction is direct or additional proteins are involved. It is interesting to note that we detected increased binding of CRB3-CLPI to importin β-1 after release of mitotic arrest, suggesting that a posttranslational modification such as phosphorylation might be involved.

We also observed that the interaction of CRB3-CLPI with importin β-1 was regulated by Ran GTP. As has been found with other importin β-1 interactions, Ran GTP weakened the interaction between importin β-1 and this cargo. Ran–importin β complexes play a fundamental role during mitosis, including targeting spindle assembly factors (Nachury et al., 2001; Wiese et al., 2001; Ciciarello et al., 2004; Kalab et al., 2006; Sillje et al., 2006; Silverman-Gavrila and Wilde, 2006). Ran is also concentrated at centrosomes and is thought to regulate centrosome cohesion, as overexpression of RanBP1 leads to abnormal centriole splitting (Di Fiore et al., 2003). The Ran network also regulates centrosome duplication and spindle assembly (Di Fiore et al., 2004; Wang et al., 2005; Silverman-Gavrila and Wilde, 2006). These defects in centrosome duplication and cohesion can lead to the multiple spindle poles seen in cells with perturbed Ran signaling. Consistent with these results are the findings that importin β-1 overexpression also leads to abnormal spindles, possibly because of defects in centriole cohesion (Di Fiore et al., 2004). Multiple spindle poles and supernumerary centrioles were also seen with loss of CRB3-CLPI from cells by shRNA, an effect that was rescued by the reexpression of CRB3-CLPI. These results suggest that CRB3-CLPI can be delivered to spindle poles by importin β-1 during mitosis, and this delivery may be important for centrosome maintenance, in addition to concentrating specific membrane components near the centrosome. Although it is intriguing to implicate complex mechanisms of centrosome maintenance as the cause of the supernumerary centrosomes, it is also likely that many of the cells had supernumerary centrosomes as a result of cytokinesis defects. Indeed, there was a strong correlation between multiple nuclei and supernumerary centrosomes in our studies, suggesting that cytokinesis defects could have played a large role in the centrosome abnormalities. In fact, we saw multiple centrosomes in almost all multinuclear cells. However, there were ∼12% of cells that had a single nucleus and supernumerary centrosomes, suggesting that CRB3-CLPI knockdown might have a direct effect on centrosomes in addition to the cytokinesis defect.

We also detected importin β-1 with CRB3-CLPI in the cilia by immunostaining. Indeed, proteomic studies have identified importin family members in the centrosome and cilia (Andersen et al., 2003; Pazour et al., 2005). The finding of a connection between nuclear proteins, cilia, and centrosomes described in this paper is not unique (Khanna et al., 2005). It was especially interesting to see the loss of cilia in cells expressing dominant-negative importin. Recently, a hypothesis was generated suggesting that there may be similarities between the nuclear pore complex proteins and intraflagellar transport proteins (Jekely and Arendt, 2006). Importins that interact with the nuclear pore complex might also have similar types of interactions with the intraflagellar transport complex delivering cilia proteins such as CRB3-CLPI.

The finding of importin proteins in the cilia is also of great interest because of recent data demonstrating the signaling pathways that lead from the cilia to the nucleus. The best documented of these is the hedgehog pathway that leads to processing of gli transcription factors (Huangfu and Anderson, 2005). It has been suggested that this processing may occur in the cilia, and the processed gli products would need to be sent to the nucleus (Haycraft et al., 2005). Several other cilia to nuclear signaling pathways have been described necessitating the need for the trafficking of proteins from the cilia to the nucleus (Chauvet et al., 2004; Low et al., 2006). Therefore, importins may have a role transporting proteins from the cilia directly to the nucleus. In summary, our findings describe a unique membrane compartment containing CRB3-CLPI that lies close to centrosomes during cell division and ciliogenesis. They also indicate an important role for this membrane compartment not only in ciliogenesis but also in cell cycle control and possibly polarity determination. Finally, they point to a new role for the multipurpose importin family in delivering cellular components to the centrosome.

Materials and methods

Plasmid construct

CRB3-ERLI constructs were previously described (Makarova et al., 2003; Fan et al., 2004). For expression of Myc–CRB3-CLPI, full-length CRB3-CLPI was amplified from a human embryo cDNA library and cloned into pcDNA3.1 Zeo (+) vector via BamH and Not1 sites. Then, using single primer mutagenesis, a single Myc tag was placed behind the signal peptide (Makarova et al., 2000). To rescue CRB3-CLPI shRNA clones in MDCK cells, we deleted the CMV promoter of pcDNA3.1 Myc–CRB3-CLPI to decrease the expression level of the transfected construct. For expression of

Flag–importin β-1 wt and Flag–importin β-1 N-deletion, we amplified human full-length importin β-1 from an EST clone (American Type Culture Collection) and subsequently ligated the amplified product to p3xFLAG-CMV-9 vector and pGSTag vector (Sigma-Aldrich) via a BamH1 site. Then, we deleted the first 360 amino acids of importin β-1 full length to generate Flag–importin β-1 N-deletion. Full-length human GTPase Ran was amplified from an EST clone (American Type Culture Collection) and subsequently ligated to pcDNA3.1 Zeo(+) vectors via BamHI and Xho1 sites. Ran Q69L and Ran T24N were generated by mutagenesis using single primers (Makarova et al., 2000).

Cell culture

MDCK II, HeLa, and COS-7 cells were cultured as described previously (Hurd et al., 2003; Roh et al., 2003). MDCK cells were transfected with Myc–CRB3-CLPI or Myc–CRB3-ERLI (FuGENE 6 transfection Reagent; Roche) and cultured in DME complete media supplemented with 200 μg/ml Zeocin (Invitrogen) for 10–14 d, and clones were selected. Flag–importin β-1 N-del stable cell lines were cultured in DME media with 600 μg/ml G418 to obtain clones.

shRNA

Double-stranded oligonucleotides corresponding to canine CRB3-CLPI 3′ nontranslated sequences TAGCAGGGAAGAAGGTACT and GAAGGTACTTCAAAGACTC were selected for CRB3-CLPI shRNA targeting sequences and inserted into the pSilencer vector (Ambion). Stable knockdown clones were selected in 200 μg/ml Hygromycin B. CRB3-ERLI shRNA stable knockdown clones were selected as described for CRB3-CLPI shRNA clones using the canine targeting sequence of CCTCAAGCTGCCACCCGAG. Double-stranded oligonucleotides corresponding to canine importin β-1 sequences ACCCCAACAGCACAGAGCA and GAGGATGCCCTGATAGCAG were selected as importin β-1 shRNA targeting sequences using the pSilencer vector. Importin β-1 transient knockdown was induced by importin β-1 shRNA transfection using Lipofectamine 2000 (Invitrogen) for 48 h.

Immunostaining and confocal microscopy

We performed immunostaining as described previously (Fan et al., 2004). Rabbit anti–CRB3-CLPI was made against peptides of NHAAEARAPQDSKETVRGCLPI. Mouse anti–Flag M2, mouse anti–acetylated tubulin, mouse anti–α-tubulin, mouse anti–γ-tubulin (Sigma-Aldrich), rat anti–α-tubulin (Chemicon), mouse anti–importin β-1 (ABR Affinity BioReagents and BD Biosciences), mouse anti–importin α/Rch-1, mouse anti-Ran, mouse anti-EEA1, mouse anti-Rab11, mouse anti-GM130 (BD Biosciences), rabbit anti-Giantin, and rabbit anti-pericentrin (Covance) were used for immunofluorescence or immunoblots. Rabbit anti–CRB3-ERLI was as previously described (Makarova et al., 2003). All images were obtained using a meta laser-scanning confocal microscope (LSM 510; Carl Zeiss MicroImaging, Inc.). Samples were scanned with appropriate lasers and filter sets, and images were collected at 0.5-μm intervals on an inverted microscope (Axiovert 100M; Carl Zeiss MicroImaging, Inc.) using a 63× water objective (C-Apochromat) with 1.2 NA. LSM 510 meta software (Carl Zeiss MicroImaging, Inc.) was used to collect images. Images were analyzed with LSM image browser (Carl Zeiss MicroImaging, Inc.), and subsequent preparation was performed using Creative Suite software (Adobe). 2D images were taken using a 60× oil objective with 1.4 NA (Plan Apo) on an inverted microscope (Eclipse TE2000U; Nikon). Image acquisition was performed with MetaMorph software and a charge-coupled device camera (Carl Zeiss MicroImaging, Inc.).

Immunoprecipitation and Western blotting

Lysis buffer (50 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton, and 10% glycerol) with protease inhibitor cocktail tablets (Roche) and phosphatase set I and II (EMD Bioscience) was used to extract cells. Antibodies to Myc 4A6 (Upstate Biotechnology), Flag M2 (Sigma-Aldrich), or importin β-1 (BD Biosciences) were added to Cos-7, HeLa, or MDCK cell extracts overnight at 4°C. 50 μl of 50% protein A/G beads (Zymed Laboratories) was added to the lysate for 2 h to bind the antibodies. After washing, the immunoprecipitates were eluted with sample buffer, separated by Bis-Tris PAGE, transferred to nitrocellulose, and immunoblotted (Hurd et al., 2003). Large-scale anti-Myc immunoprecipitation of Myc–CRB3-CLPI and Myc–CRB3-ERLI MDCK stable cells was performed as described previously (Roh et al., 2002). The specific bands that coimmunoprecipitated with Myc–CRB3-CLPI were cut from the gel and analyzed at the Michigan Proteome Consortium using a 4800 Proteomic Analyzer (Applied Biosystems).

Calcium switch experiments

MDCK II, CRB3-CLPI, and CRB3-ERLI shRNA MDCK stable cells were grown on transwell filters until confluent. After washing with cold PBS (without calcium) three times, low calcium media (5 μM Ca^{2+}) was added to the cells overnight. The next day, DME complete media (2 mM Ca^{2+}) was added to the cells that were then fixed and stained at the time points indicated (Roh et al., 2003).

Synchronization of HeLa cells

For synchronizing HeLa cells, 100 ng/ml Nocodazole (Sigma-Aldrich) in DME complete media was added to the cells for 12 h. After washing three times with ice-cold PBS, cells were placed in warm DME complete media and lysed 30 min later.

Online supplemental material

Fig. S1 shows that the CRB3-CLPI antigenic peptide blocks the anti–CRB3-CLPI staining of both endogenous and transfected proteins, CRB3-CLPI localizes to spindle poles and the midbody during mitosis in COS-7 cells, and CRB3-ERLI does not localize to the spindle poles during mitosis. Fig. S2 shows that overexpression of Myc–CRB3-ERLI does not induce the multinuclear phenotype in MDCK cells. Fig. S3 shows that after growth arrest, CRB3-CLPI knockdown cells did display multinuclei and supernumerary centrosomes. Fig. S4 shows that an importin β-1 GST fusion protein is able to precipitate Myc–CRB3-CLPI. Fig. S5 shows that the Flag–importin β-1 N-deletion mutant protein colocalizes with CRB3-CLPI in spindle poles during mitosis in MDCK cells, and CRB3-CLPI does not colocalize with GM130, EEA-1, or Rab11. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200609096/DC1.

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