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Actin cytoskeleton remodeling during early *Drosophila* furrow formation requires recycling endosomal components Nuclear-fallout and Rab11

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Cytokinesis requires a dramatic remodeling of the cortical cytoskeleton as well as membrane addition. The *Drosophila* pericentrosomal protein, Nuclear-fallout (Nuf), provides a link between these two processes. In *nuf*-derived embryos, actin remodeling and membrane recruitment during the initial stages of metaphase and cellular furrow formation are disrupted. Nuf is a homologue of arfophilin-2, an ADP ribosylation factor effector that binds Rab11 and influences recycling endosome (RE) organization. Here, we show that Nuf is an important component of the RE, and that these phenotypes are a consequence of Nuf activities at the RE. Nuf exhibits extensive colocalization with Rab11, a key RE component. GST pull-downs and the presence of a conserved Rab11-binding domain in Nuf demonstrate that Nuf and Rab11 physically associate. In addition, Nuf and Rab11 are mutually required for their localization to the RE. Embryos with reduced levels of Rab11 produce membrane recruitment and actin remodeling defects strikingly similar to *nuf*-derived embryos. These analyses support a common role for Nuf and Rab11 at the RE in membrane trafficking and actin remodeling during the initial stages of furrow formation.

Introduction

The production of two daughter cells at the end of mitosis is accomplished through a dramatic constriction of the plasma membrane. This is known as cytokinesis and involves the formation of an actin/myosin-based contractile ring that forms perpendicular to and midway between the anaphase spindle (for reviews see Fishkind and Wang, 1995; Field et al., 1999; Robinson and Spudich, 2000; Glotzer, 2001). In animal cells, the position of the mitotic spindle largely determines the position and orientation of the contractile ring (Scholey et al., 2003). Actin, myosin II, and other furrow components (such as anillin and the septins) are recruited to this site and form the contractile ring. Once the contractile ring forms, constriction of the plasma membrane occurs.

Although the mechanism of constriction is contractile, recent reports have begun to define the role of membrane addition in this process (Finger and White, 2002). A cell undergoing cytokinesis requires significant additional membrane to accommodate the increased surface area of producing two daughter cells. Work in *Xenopus* relying on a variety of surface-marking techniques indicates that the additional membrane has a different composition from the original membrane (Kalt, 1971; Bluemink and de Laat, 1973; Byers and Armstrong, 1986; Bieliavsky et al., 1992). This suggests that the membrane is not derived from the expansion of preexisting surface membrane, but instead forms through insertion of membrane from internal stores. In plant cells, it is well established that the additional membrane necessary for cytokinesis is provided through a Golgi-based delivery system (Bednarek and Falbel, 2002). In *Caenorhabditis elegans* ovaries, RNA interference inhibition of Rab11, the small GTPase required for vesicle transport through the recycling endosome (RE), causes cytokinesis defects including furrow

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Abbreviations used in this paper: Arf, ADP ribosylation factor; Arfo2, arfophilin-2; Dah, discontinuous actin hexagon; Nuf, Nuclear-fallout; RE, recycling endosome.

regression and scission (Skop et al., 2001). Mutation and RNA interference analyses demonstrate that the t-SNARE syntaxin 1 is required for cytokinesis during early embryogenesis (Burgess et al., 1997; Conner and Wessel, 1999; Jantsch-Plunger and Glotzer, 1999). Lamellar bodies, the ER, and internal lipid stores may also prove important in providing membrane for cytokinesis furrows (Fullilove and Jacobson, 1971; Bluemink and de Laat, 1973; Sanders, 1975; Leaf et al., 1990).

The rapid and simultaneous formation of thousands of furrows during early *Drosophila* embryogenesis makes this system particularly valuable for studying the recruitment of membrane and other furrow components during cytokinesis. *Drosophila* development begins with 13 synchronous, rapid, syncytial nuclear divisions. After nine divisions in the interior of the embryo, divisions 10–13 occur in the actin-rich cortex, just beneath the plasma membrane (Foe and Alberts, 1983). The nuclei and their associated centrosomes induce a dramatic redistribution of the cortical actin. During interphase, actin concentrates into caps centered above each cortical nucleus and its apically positioned centrosomes. As the nuclei progress into prophase, the centrosomes migrate toward opposite poles and the actin caps undergo a dramatic redistribution to form an oblong ring outlining each nucleus and its associated separated centrosome pair (Karr and Alberts, 1986; Kellogg et al., 1988). These rings are equivalent in composition to conventional cytokinesis contractile rings and include actin, myosin II, spectrins, cofilin, ARP, anillin, septins, and formins (Miller and Kiehart, 1995; Stevenson et al., 2002). In addition, these components are closely associated with the plasma membrane and are required for the invagination of these rings around the spindles. These rings are referred to as metaphase or pseudocleavage furrows (Schejter and Wieschaus, 1993; Sullivan and Theurkauf, 1995). At metaphase, the furrows invaginate to a depth of ~8 μm to form a half shell that encompasses each spindle. During late anaphase and telophase, the metaphase furrows rapidly regress. Centrosome duplication occurs during late anaphase, and the newly formed centrosome pairs locate apically. The actin caps reform directly above the centrosome pairs in the next interphase. This alternation between interphase actin caps and metaphase furrows occurs until interphase of nuclear cycle 14. At this point, the nuclei remain in interphase and an inverted microtubule basket, which originates from an apically positioned centrosome pair, guides invagination of the cellularization furrows (for review see Schejter and Wieschaus, 1993). At a depth of 35 μm, the furrows pinch off at their base to form individual mononucleate cells.

Genetic and biochemical analyses indicate that vesicle fusion plays an important role in furrow formation in early *Drosophila* embryogenesis. Mutations in dynamin, a GTPase involved in endocytic vesicle formation, disrupt cellular furrow formation and result in an abnormal accumulation of vesicles in the cytoplasm (Swanson and Poodry, 1981). Unconventional myosin VI has been shown to be involved in the transport of cytoplasmic particles in the *Drosophila* embryo, and mutations in this gene cause defects in formation of the metaphase furrows (Mermall et al., 1994; Mermall and Miller, 1995). α-Adaptin, a coated vesicle component necessary for receptor-mediated endocytosis, is concentrated apically and laterally around the metaphase and cellularization furrows (Dornan et al., 1997). Syntaxin 1, a t-SNARE involved in vesicle targeting, is also required for cellularization in *Drosophila* (Burgess et al., 1997). Inhibition of Golgi-based vesicle transport inhibits progression of the cellularization furrow front (Sisson et al., 2000). In addition, a major source of this membrane necessary for the cellularization furrows is derived internally rather than from the plasma membrane (Lecuit and Wieschaus, 2000).

Activities associated with the centrosome are also important for vesicle-mediated metaphase and cellular furrow formation. Insights into the centrosome-associated activities directing these rearrangements have come from the analysis of the maternal effect mutation, *nuclear fallout* (*nuf*). Nuf encodes a pericentrosomal protein that is essential for normal metaphase and cellularization furrow formation. Nuf concentrates at the centrosomes during prophase, when metaphase furrows are forming (Rothwell et al., 1998). In the *nuf* mutation, microtubule dynamics and distribution appear normal, but remodeling and recruitment of actin to the furrows is disrupted and actin remains abnormally concentrated around the centrosomes. Vesicle-based membrane recruitment to the furrows is also disrupted in *nuf*-derived embryos (Rothwell et al., 1999; Zhang et al., 2000). These phenotypes lead to the intriguing suggestion that a common mechanism mediates actin remodeling and membrane addition during cytokinesis.

Here, we provide additional insight into these two processes by demonstrating that Nuf is a component of the RE and *nuf* phenotypes are a consequence of Nuf activities at the RE. Nuf exhibits extensive colocalization with Rab11, a member of the Rab family of small GTPases specific to the RE (Mellman, 1996; Ullrich et al., 1996). In addition, Rab11 and Nuf exhibit a mutual dependence for their normal localization to the RE. Rab11-deficient embryos produce metaphase and cellular furrow defects strikingly similar to those observed in *nuf*-derived embryos. In accord with these results, recent reports demonstrate that Nuf is a homologue of arfophilin-2 (Arfo2), an ADP ribosylation factor (Arf) effector that also binds Rab11 and influences RE organization (Hickson et al., 2003). Together, these reports suggest that actin remodeling during the initial stages of cytokinesis may in part rely on endosomal-mediated membrane delivery to the site of furrow formation.

Results

Live analysis of Nuf reveals dynamic flares and puncta radiating from the centrosome

Previous immunofluorescent analyses demonstrated that Nuf concentrates at the centrosomes during prophase and diffusely localizes throughout the cytoplasm during the remainder of the cell cycle (Rothwell et al., 1998). To visualize the cell cycle dynamics of Nuf in real time, we constructed a GFP-Nuf transgenic line (see Materials and methods). The GFP-Nuf construct completely rescues *nuf*-induced maternal lethality. Fig. 1 A presents live analysis of a GFP-Nuf–expressing embryo (green) through nuclear cycle 12 injected with fluorescently labeled tubulin (red). During interphase,

Nuf accumulates at each of the separating centrosomes. During prophase, immediately before nuclear envelope breakdown, Nuf accumulation peaks, concentrating around the base of the astral microtubules radiating away from the centrosomes. Nuf is absent on the side of the centrosome adjacent to the nuclear envelope. Significantly, maximal Nuf localization at prophase corresponds to the time of metaphase furrow invagination. Nuf localization is correlated with areas of high astral microtubule density. This is in accord with our finding that Nuf pericentriolar localization requires intact microtubules (unpublished data). Although the pericentriolar concentration of Nuf significantly diminishes during metaphase and anaphase, a small fraction of Nuf remains tightly associated with the centrosomes. During telophase, immediately after nuclear envelope reformation, Nuf begins accumulating at the newly duplicated centrosome pair. The low magnification images depicted in Fig. 1 B dramatically highlight the cell cycle regulation of Nuf subcellular localization. We do not know if Nuf is maintained in constant levels throughout the nuclear cycle and is

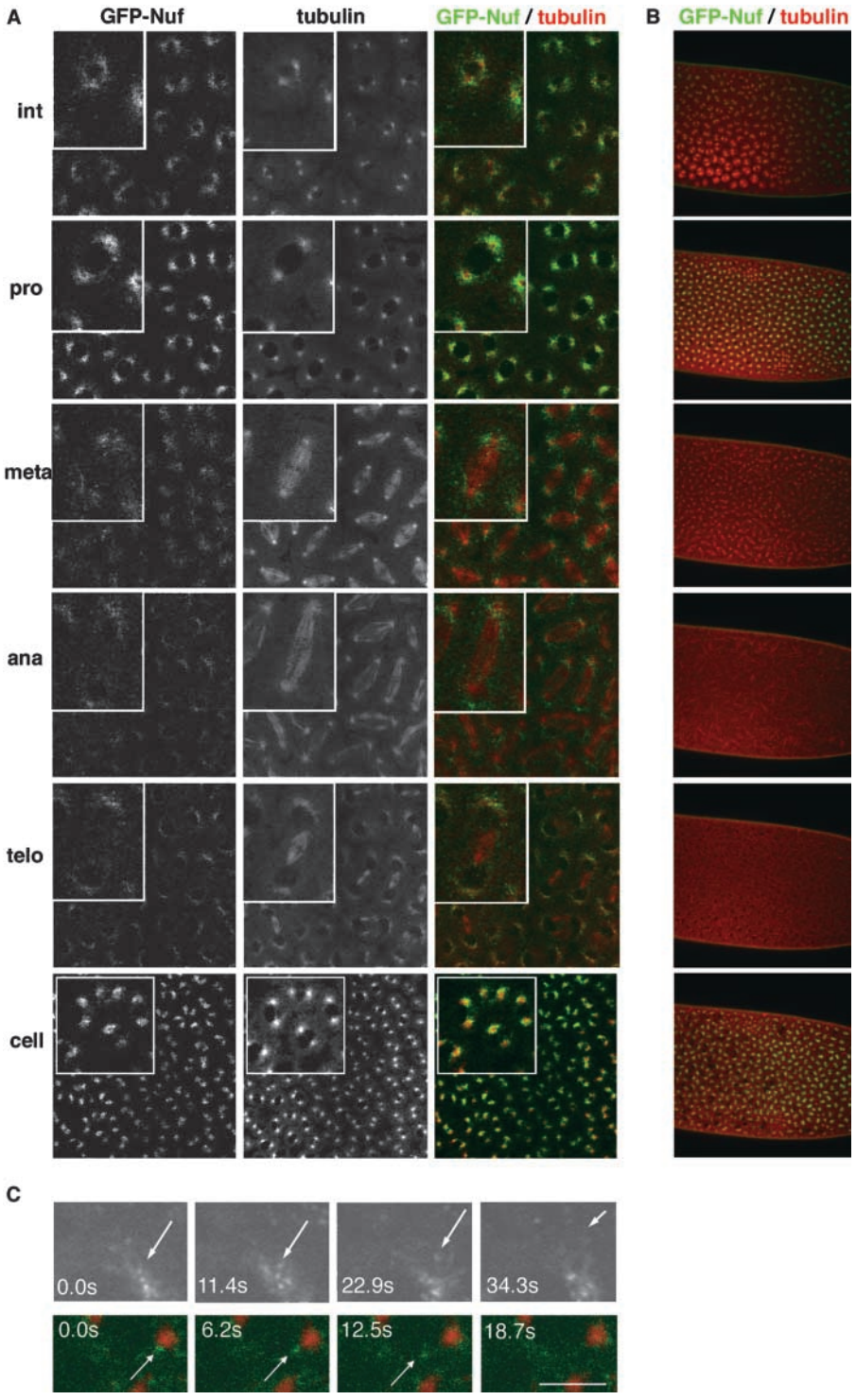

Figure 1. **Nuf concentrates at the centrosomes during prophase.** (A) Live analysis of GFP-Nuf (green) and tubulin (red) during the cortical divisions. Nuf accumulates at the centrosome during interphase and reaches a maximum during prophase. As the nuclei progress into metaphase, Nuf becomes diffusely localized in the cytoplasm with only a small amount remaining at the centrosomes. Immediately after nuclear envelope formation at telophase, Nuf begins accumulating at the centrosomes. During the prolonged interphase of nuclear cycle 14, as cellularization is occurring, Nuf reaches high levels of accumulation at the centrosomes. These levels are maintained until the end of cellularization. (B) Low powered images of a GFP-Nuf (green) transgenic embryo injected with rhodamine-labeled tubulin (red) throughout the cortical nuclear cycle. Images highlight the dramatic cycling of Nuf levels at the centrosome. (C) Nuf is highly dynamic at the centrosomes. Top: spinning confocal images of a GFP-Nuf transgenic embryo during prophase of the cortical syncytial divisions. Nuf is extremely dynamic, forming flares and puncta that rapidly migrate away from the centrosome. The arrows follow the formation of a Nuf particle as it travels away from the centrosome. Bottom: confocal images of a GFP-Nuf (green) transgenic embryo injected with rhodamine-labeled tubulin (red). These images demonstrate that migration of Nuf particles is characterized by stationary periods (see arrows in first two panels) followed by rapid migration. Insets, 2×. Bar, 5 μm.

simply cycling from the cytoplasm to the centrosomes, or if Nuf levels change throughout the cell cycle. The localization of Nuf during cellularization at nuclear cycle 14 differs significantly from its localization during the syncytial divisions. During the syncytial divisions, Nuf is present in lower levels at the centrosome during interphase (Fig. 1 A, top row), reaches its maximal concentration, and is highly dynamic during prophase. In contrast, during cellularization, Nuf reaches its maximal concentration during interphase and is relatively motionless, forming few flares and puncta (Fig. 1 B, bottom row). During the syncytial divisions, Nuf concentrates only in the region of the centrosome facing away from the nuclear envelope. However, during cellularization, Nuf is more evenly distributed around the centrosome, forming an intact ring. The differences between Nuf behavior during the syncytial divisions and cellularization may be a consequence of the more stable microtubule arrays that form during the prolonged interphase of nuclear cycle 14.

Nuf is extremely dynamic at the centrosome during prophase. Detailed imaging reveals that Nuf forms dynamic puncta and flares that rapidly migrate from the centrosomes. The arrows in Fig. 1 C follow the formation of a Nuf particle traveling away from the centrosome. The puncta form, travel a short distance from the centrosome, then disappear. As described below, Nuf is associated with the recycling endosomal compartment. Therefore, this movement may reflect endosomal dynamics (Sonnichsen et al., 2000).

Rab11 localizes at the centrosome in the early *Drosophila* embryo

The mammalian homologue of Nuf, Arfo2 physically associates and colocalizes with Rab11, a key component of the RE (Hickson et al., 2003). Rab11 is required for the integrity of the RE, and is believed to mediate transport of vesicles from the RE to the TGN, early endosome, and plasma membrane via a "slow" recycling route (Ullrich et al., 1996; Ren et al., 1998). Dollar et al. (2002) characterized the pattern of Rab11 localization in the developing *Drosophila* oocyte. They demonstrated that Rab11 localizes at the posterior pole and is necessary for proper microtubule organization and Oskar mRNA localization. Here, we examine the pattern of Rab11 localization during the cortical divisions in the early *Drosophila* embryo. Shown in Fig. 2 are triple-stained immunofluorescent images of Rab11 (green), the centrosomal protein centrosomin (Cnn; red), and DNA (blue) during nuclear cycle 12. During interphase, Rab11 exhibits a diffuse punctate localization that concentrates around the nuclei. As the embryos progress into prophase, Rab11 maintains its punctate morphology, but exhibits significantly increased concentration at the centrosomes. During metaphase, the centrosomal concentration of Rab11 decreases and there is a concomitant dispersal of Rab11 throughout the cytoplasm encompassing each chromosome–spindle complex. This trend continues as the nuclei enter anaphase. Even though the nuclear envelope is substantially broken down during metaphase and anaphase, Rab11 does not enter the interior nuclear space. During telophase, Rab11 puncta concentrate around the newly formed nuclear envelope. There is a slight increase in the concentration of

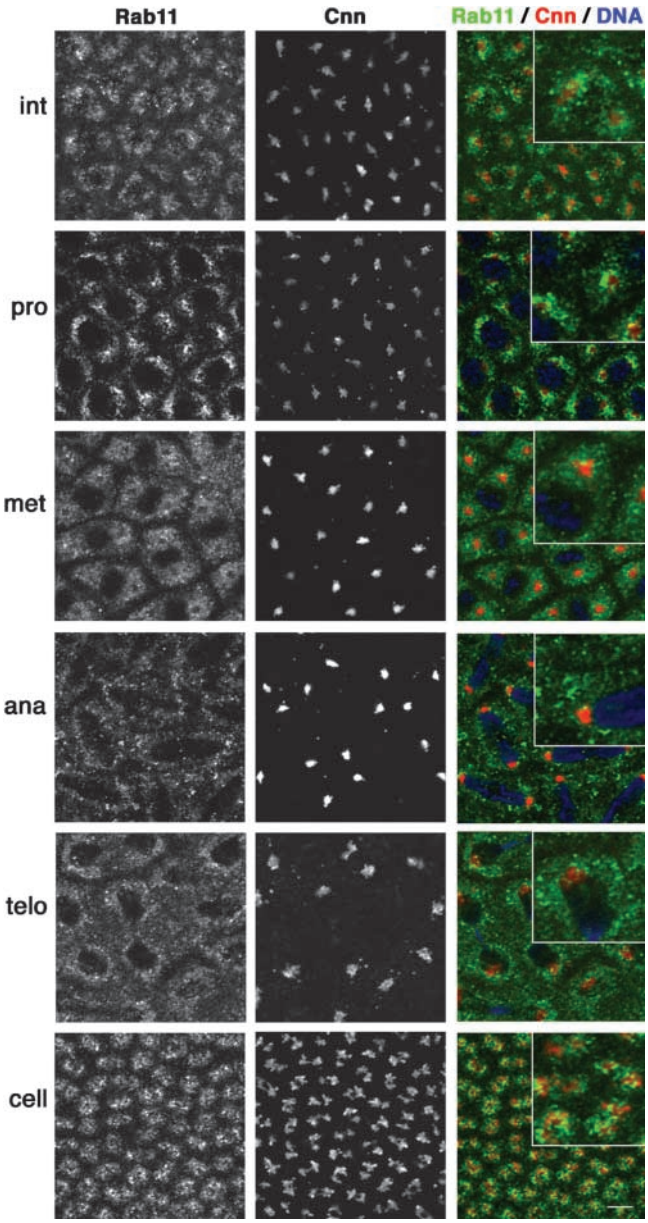

Figure 2. **Pericentriolar Rab11 localization during cortical divisions.** Cortical nuclear cycle 12 and cellularizing embryos were triple stained for Rab11 (green), Cnn (red), and DNA (blue). The pericentriolar concentration of Rab11 is most pronounced during prophase of the cortical divisions and during interphase of nuclear cycle 14 when cellularization is occurring. Insets, 2×. Bar, 5 μm.

Rab11 puncta at the centrosomes. Cellularization occurs during the prolonged interphase of nuclear cycle 14. At this time, Rab11 is highly concentrated around the pair of apically located sister centrosomes.

The pericentriolar concentration of Rab11 in *Drosophila* embryos is equivalent to Rab11 localization observed in mammalian cells. In CHO cells, Rab11 is primarily localized to a discrete pericentriolar region with a lower concentration of puncta distributed throughout the cell (Ullrich et al., 1996). Colocalization experiments with internalized transferrin indicated that Rab11 localizes to the pericentriolar RE (Ullrich et al., 1996; Sheff et al., 2002). GFP-Rab11

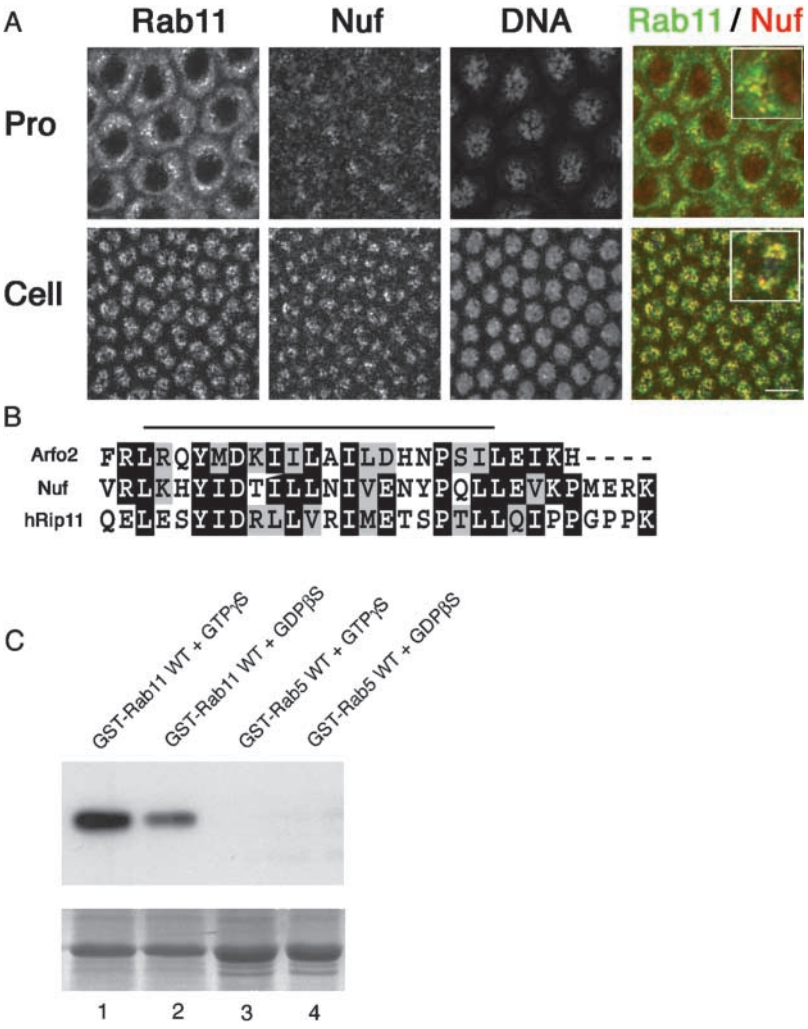

Figure 3. **Rab11 and Nuf interact.** (A) Cortical nuclear cycle 12 and cellularizing embryos were triple stained for Rab11 (green), Nuf (red), and DNA. Rab11 and Nuf colocalize during prophase and cellularization when both antigens are highly concentrated in the pericentriolar region (insets, yellow spots). Insets, 2×. Bar, 5 μm. (B) Nuf contains a Rab11-binding domain. Sequence alignment of the COOH-terminal ends of Arfo2, Nuf, and hRip11. Line denotes the 20-aa domain critical for Rab11 binding. (C) Nuf and Rab11 are able to physically interact. GST pull-downs demonstrate a physical interaction between Rab11 and Nuf. Glutathione-Sepharose beads loaded with equal amounts of wild-type GST-Rab11 (lane 1 and lane 2) or GST-Rab5 (lane 3 and lane 4) were incubated with equal amounts of a CHO cell lysate containing overexpressed GFP-Nuf in the presence of 10 μM GTPγS or GDPβS. After incubation as described, the beads were recovered and immunoblotted for Nuf (top). The bottom panel shows an SDS-PAGE analysis of the GST-Rab11 and -Rab5 used in the experiment, and illustrates that roughly equal amounts of GST-Rab were loaded in all four lanes. The data shown are representative of three experiments of this type.

also exhibits a pericentriolar localization and colocalizes with the transferrin receptor (Sonnichsen et al., 2000). Given the equivalent staining patterns, we conclude that Rab11 also localizes to the RE in syncytial and cellularized *Drosophila* embryos.

Nuf and Rab11 colocalize at the centrosome

We performed immunofluorescent analyses using anti-Nuf (red) and anti-Rab11 (green) antibodies (Fig. 3 A). During prophase, when both antigens are highly concentrated in the pericentriolar region, areas of maximal Nuf localization correspond to areas of maximal Rab11 localization (yellow spots). Almost without exception, Nuf colocalizes with Rab11 (inset; few if any red puncta). However, the converse is not true, and in regions more distal from the centrosome, Rab11, but not Nuf, is present (inset; numerous green puncta). During cellularization at interphase of nuclear cycle 14, Nuf and Rab11 exhibit high pericentriolar concentrations and extensive colocalization. As observed for prophase of the cortical divisions, Nuf always colocalizes with Rab11, but there are regions of Rab11 localization in which Nuf is not present. Given that Rab11 is an excellent marker of the RE (Ullrich et al., 1996; Ren et al., 1998), these results support the notion that Nuf localizes to the RE during cortical syncytial divisions and during cellularization at interphase of nuclear cycle 14.

Rab11 and Nuf physically associate

Nuf is a structural and functional homologue of Arfo2 (Hickson et al., 2003) and contains a highly conserved 20-aa Rab11-binding site (Fig. 3 B). This binding domain was first identified by Prekeris et al. (2001) and Hales et al. (2001) as important for the interaction between Rab11 and a novel family of putative Rab11 effector proteins. Within this domain, Nuf and Arfo2 contain eight identical and six conserved amino acids. Nuf and hRip11, a mammalian Rab11 effector protein, contains ten identical and three conserved amino acids. This sequence conservation, combined with the colocalization results, prompted us to examine whether Nuf and Rab11 physically interact. Bacterially expressed GST-Rab11 was mixed with CHO cells transiently expressing GFP-Nuf. GTPγS and GDPβS were added to the buffer to test the nucleotide specificity of the interaction. As shown in Fig. 3 C, GFP-Nuf was effectively pulled down by both GST-Rab11+GTPγS and GST-Rab11+GDPβS, indicating that the interaction is not tightly linked to the state of the nucleotide (lane 1 and lane 2). GST-Rab11+GDPβS pulled down Nuf to a lesser extent than

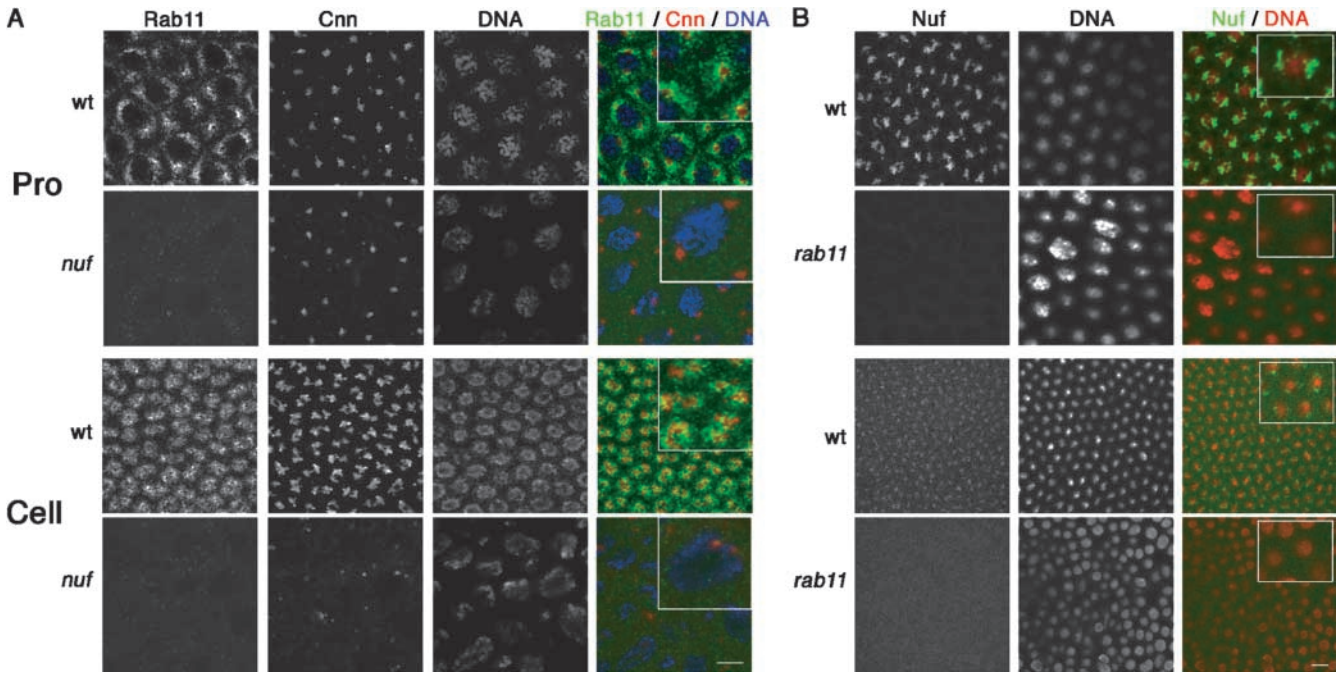

Figure 4. **Interdependence of Nuf and Rab11.** (A) Cortical nuclear cycle 12 and cellularizing wild-type and *nuf*-derived embryos were triple stained for Rab11 (green), Cnn (red), and DNA (blue). In wild-type embryos, high levels of Rab11 concentrate around Cnn, a centrosomal marker. In *nuf*-derived embryos, Rab11 fails to concentrate at the centrosome. (B) Cortical nuclear cycle 12 and cellularizing wild-type and *rab11*-derived embryos were double stained for Nuf (green) and DNA (red). In wild-type embryos, high levels of Nuf concentrate around the pericentrosomal area at the poles of each nucleus. In *rab11*-derived embryos, Nuf fails to concentrate around the pericentrosomal area. Insets, 2×. Bars, 5 μm.

GST-Rab11+GTPγS. Nucleotide-independent binding has also been observed with other Rab11 effectors, as Rab11-FIP2 (Hales et al., 2001) and Arfo2, the mammalian homologue of Nuf (Hickson et al., 2003). To test the specificity of the interaction, similar pull-down experiments were performed with Rab5, a component of the early endosome (Woodman, 2000). Unlike the results with GST-Rab11, GFP-Nuf is not pulled down by GST-Rab5 in either the activated or unactivated form (lane 3 and lane 4).

Functional interactions between Nuf and Rab11

To determine if Nuf is required for pericentriolar Rab11 localization, we examined Rab11 localization in *nuf*-derived embryos (Fig. 4 A). Wild-type and *nuf*-derived embryos were triple stained for Rab11 (green), Cnn (red), and DNA (blue). Rab11 exhibits a concentrated punctate distribution around the centrosome during prophase (Fig. 4 A, top row). In *nuf*-derived embryos, both the punctate distribution and concentration of Rab11 around the centrosomes is completely abolished (Fig. 4 A, second row). Although levels of Nuf at the centrosome are greatly reduced during metaphase, Nuf is required for Rab11 centrosome localization at this stage as well (unpublished data). Nuf is also required for Rab11 localization during cellularization. The robust tight localization of Rab11 around the centrosome during cellularization is absent in *nuf*-derived embryos (Fig. 4 A, bottom row). We believe the mislocalization of Rab11 in *nuf* is not a result of a general disruption of the intracellular transport pathway, as staining with Golgi marker Lavalamp (Sisson et al., 2000) revealed normal Golgi distribution throughout the cell cycle in wild-type and *nuf*-derived embryos (unpublished data). From this analysis, we cannot determine whether levels of Rab11 protein are reduced in *nuf*-derived embryos.

Also, we analyzed whether Rab11 is required for normal pericentriolar Nuf localization. Because Rab11 is an essential gene, we used a combination of hypomorphic *rab11* alleles that permitted normal zygotic development (Jankovics et al., 2001). However, these transheterozygote females produced embryos with reduced levels of maternally supplied Rab11 and showed a reduced hatch rate. Wild-type and *rab11*-derived embryos were double stained for Nuf (green) and DNA (red), and were examined during the syncytial divisions and cellularization. During prophase, while the pericentriolar localization of Nuf was robust in control embryos, pericentriolar Nuf levels were absent in *rab11*-derived embryos (Fig. 4 B, second row). We obtained the same result when we examined cellularizing *rab11*-derived embryos; the normal pericentriolar localization of Nuf is completely abolished (Fig. 4 B, bottom row). From this analysis, we cannot determine whether levels of Nuf protein are reduced in *rab11*-derived embryos. These experiments demonstrate that Nuf and Rab11 are mutually dependent on one another for their localization to the RE.

Mutations in Rab11 and Nuf exhibit similar defects in metaphase furrow formation

The *nuf* maternal-effect mutation specifically disrupts syncytial nuclear divisions only after the nuclei migrate to the cortex (Sullivan et al., 1993). These nuclear defects are a consequence of incomplete metaphase furrow formation, which allows inappropriate fusions between nonsister nuclei (Roth-

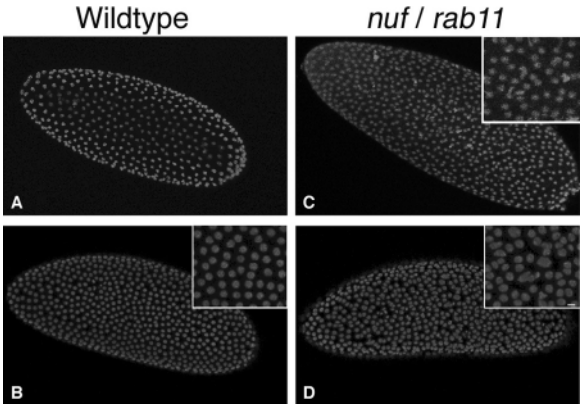

Figure 5. **Late cortical divisions disrupted in both *nuf*- and *rab11*-deficient embryos.** Embryos derived from homozygous *nuf* females and transheterozygous females bearing *rab11* mutant alleles J2D1 and 93Bi were fixed and stained for DNA. Wild-type embryos are shown during the early (A) and late (B) cortical divisions. Development from fertilization to the early cortical divisions is normal in both *nuf*- and *rab11*-deficient embryos. At cortical nuclear cycle 10, these mutations are indistinguishable from the wild type (A). However, during the late cortical divisions, *rab11*-deficient embryos exhibit severe abnormalities in nuclear spacing and morphology (inset; compare D with B). This nuclear phenotype is similar to that observed in *nuf*-deficient embryos (inset; compare C with B), and is indicative of failed furrow formation allowing inappropriate fusions between neighboring nuclei. Insets, 2×. Bar, 5 μm.

well et al., 1998). Although the interphase actin caps form normally, large gaps are present in the metaphase and cellularization furrows. The gaps are observed in the earliest stages of furrow formation, suggesting that Nuf disrupts recruitment of actin to the furrows rather than in stabilization of actin once at the furrows. To determine if reduced maternal supplies of Rab11 produced cortical phenotypes similar to those observed in *nuf* mutations, we used the *rab11* transheterozygote described above. The nuclear phenotype is equivalent to *nuf*. In *rab11*-derived embryos, nuclear distribution and morphology is normal in premigration and early cortical blastoderm embryos (Fig. 5, top row). However, during the late cortical divisions when the nuclei are more densely packed, the nuclear distribution and morphology is disrupted. In premigration and early cortical embryos, 8% (2/23) exhibit disrupted nuclear morphology. During the late cortical divisions, 65% (31/48) exhibit severely disrupted nuclear morphology. This is indicative of defects in the metaphase furrows that serve to separate neighboring nonsister nuclei (Sullivan et al., 1990).

To examine the role of Rab11 in organizing the cortical cytoskeleton and metaphase furrows, we double stained wild-type, *nuf*-derived, and *rab11*-derived cortical nuclear cycle 12 embryos for DNA (red) and actin (green). During interphase, actin organizes into caps apically positioned above each nucleus (Fig. 6 A, top row). In *nuf*- and *rab11*-derived embryos, actin cap formation occurs normally. As the embryos progress into prophase, the actin caps are dismantled and actin reorganizes into furrows encompassing each prophase nucleus and its developing spindle. As the nuclei progress into metaphase, these furrows become more pronounced and tightly focused. The actin-based furrow defects in *rab11*-derived embryos are strikingly similar to those observed in *nuf*-derived embryos. In both, the hexagonal furrow network is riddled with gaps (Fig. 6 A, middle rows, arrows). The gaps are present at prophase during the initial stages of furrow formation, suggesting defects in the initial actin recruitment. *nuf* and *rab11* mutations also produce similar defects during cellularization at nuclear cycle 14, although defects in *nuf*-derived embryos are much more extensive than observed in *rab11*-derived embryos. This difference may be a result of partial zygotic rescue by the paternally supplied rab^{+} allele (Sullivan and Pimpinelli, 1986).

rab11-derived embryos mislocalize membrane markers at the invaginating furrow

nuf-derived embryos disrupt recruitment of membrane components during furrow invagination. The *Drosophila* protein discontinuous actin hexagon (Dah) tightly associates with the plasma membrane as well as actin, and is thought to link cortical microfilaments to the plasma membrane (Zhang et al., 1996). In cortical *Drosophila* embryos, Dah localizes to the plasma membrane as well as to vesicles that concentrate at the leading edge of the invaginating furrows (Rothwell et al., 1999). Analysis of Dah mutations indicates that incorporation of these vesicles into the plasma membrane contributes to furrow invagination (Rothwell et al., 1999). To determine the role of Rab11 and Nuf in Dah-associated vesicle delivery, we double stained wild-type, *nuf*-derived, and *rab11*-derived embryos for actin (green) and Dah (red; Fig. 6 B). In *nuf*-derived embryos, incorporation of Dah into the metaphase furrows is dramatically reduced. Although Dah vesicles are observed, they are more randomly distributed throughout the cytoplasm. A similar defect is observed in *rab11*-derived embryos; incorporation of Dah into the invaginating metaphase furrows is disrupted. However, in contrast to *nuf*, Dah staining is not observed in the furrow regions and few Dah-staining vesicles are visible.

Discussion

Nuf is a homologue of Arfo2

Previous reports demonstrated that Nuf is a pericentrosomal protein required for the recruitment of both actin and membrane during furrow formation in the early *Drosophila* embryo (Rothwell et al., 1999). Further insight into Nuf action at the centrosome comes from recent reports demonstrating that the mammalian homologue of Nuf is an Arf effector protein, Arfo2 (Hickson et al., 2003). Arf proteins are members of a large family of small GTPases involved in the regulation of membrane-trafficking pathways. Arfo2 and Nuf show significant similarities at the COOH terminus (300-aa region). This region is predicted to form extensive coiled-coils and is 28% identical and 54% conserved between these two proteins. A striking feature of these proteins is that they contain a previously identified 20-aa Rab11-binding domain at their extreme COOH termini (Hales et al., 2001; Prekeris et al., 2001; see below).

Nuf and Arfo2 are functionally as well as structurally related. In HeLa cells, Arfo2 localizes to the perinuclear TGN

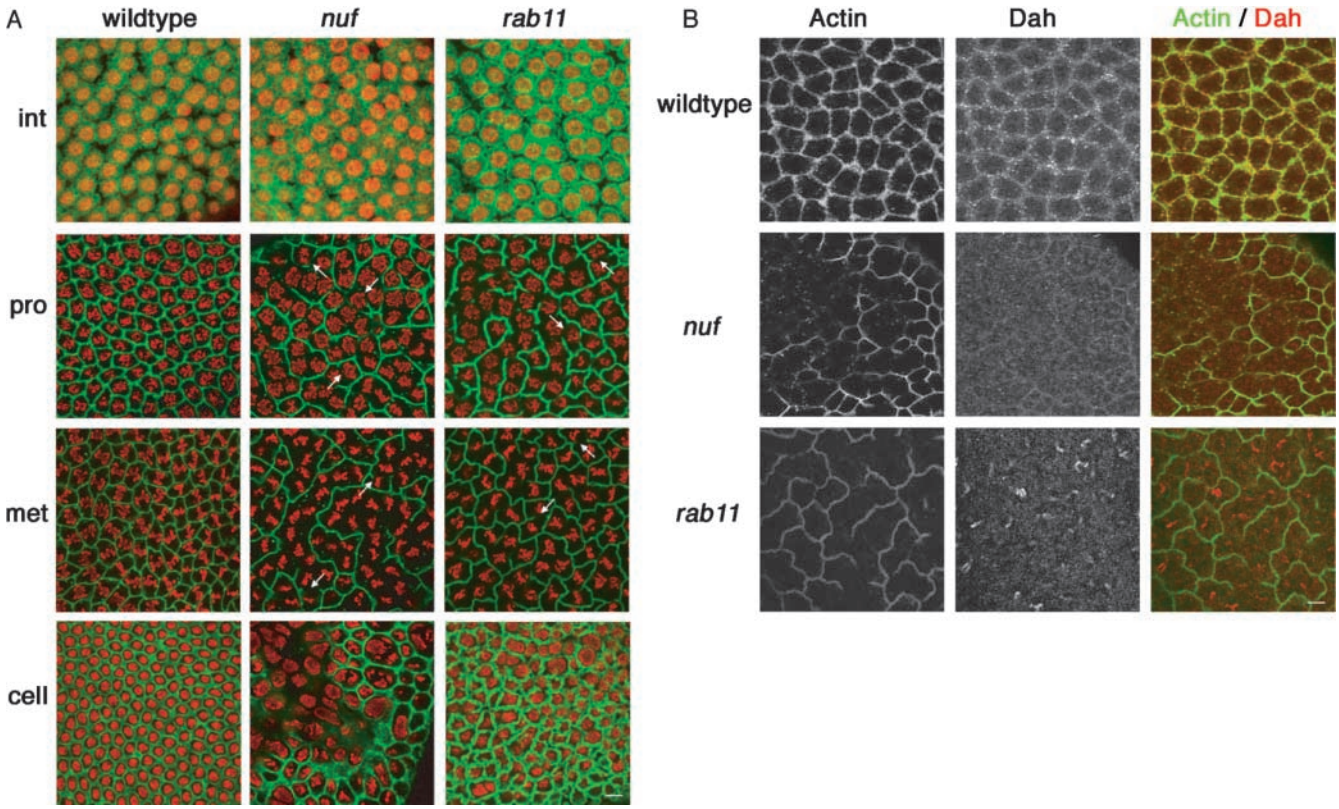

Figure 6. **Furrow and membrane defects in *nuf*- and *rab11*-derived embryos.** (A) Wild-type, *nuf*-derived, and *rab11*-derived cortical nuclear cycle 12 and cellular embryos were double stained for actin (green) and DNA (red). In both mutants, the actin caps form normally, but large actin gaps are present in the furrows as they are forming during prophase and when they are fully invaginated at metaphase (arrows). Similar defects are also observed in the cellularization furrows in *nuf*-derived embryos. The more mild defects observed in *rab11*-derived embryos at cellularization may be a consequence of partial paternal rescue. (B) Wild-type, *nuf*-derived, and *rab11*-derived embryos were double stained for actin (green) and Dah (red). Dah is closely associated with membrane and actin and is essential for furrow invagination. In both *nuf*- and *rab11*-derived embryos, Dah localization to the invaginating furrows fails. In *nuf*-derived embryos, Dah remains in punctate particles distributed throughout the cortical cytoplasm. In *rab11*-derived embryos, Dah staining is completely absent from the furrows. Bar, 5 μm.

with staining also observed at the centrosomes and focal adhesions (Hickson et al., 2003). In *Drosophila*, Nuf has a similar localization at the centrosomes (Rothwell et al., 1998). Overexpression of either *Drosophila* Nuf or human Arfo2 in mammalian cells results in a collapse of the late RE to a pericentrosomal region (Hickson et al., 2003). These observations suggest that Nuf and Arfo2 are functionally similar and play a role in maintaining the integrity of the RE.

The fact that both Nuf and Arfo2 contain a conserved Rab11-binding domain provides additional support for a common function at the RE. Similar to Arfs, Rabs are members of a large family of small GTPases involved in the regulation of vesicle-trafficking pathways (Segev, 2001). However, unlike Arfs, they are thought to be involved in vesicle targeting rather than vesicle biogenesis. Rab11 is primarily localized at the RE and plays an essential role in receptor-mediated recycling to the plasma membrane (Ullrich et al., 1996; Sheff et al., 2002). In addition, the Rab11 GTPase cycle is essential for normal RE organization and function (Ullrich et al., 1996). Sequence analysis of Arfo2 and Nuf (Fig. 3 B) reveals a common conserved 20-aa Rab11-binding domain originally identified among members of the Rab11-interacting protein family (Hales et al., 2001; Prekeris et al., 2001). In accord with this observation, Arfo2 and Nuf physically interact with Rab11 (Fig. 3 C; Hickson et al., 2003).

Nuf is closely associated with the RE

Our work indicates that Nuf is primarily associated with the RE in the early *Drosophila* embryo. Nuf shows extensive colocalization with Rab11 (Fig. 3 A). The most significant difference between the distribution of Rab11 and Nuf in the early embryo is that the former maintains a constant level of pericentriolar staining, whereas levels of the latter oscillate with the cell cycle (Fig. 1, A and B; Fig. 2). During the cortical syncytial divisions, pericentriolar Nuf staining is at its highest levels at prophase and negligible during metaphase and anaphase. We do not know whether this is a result of cycling of Nuf levels, subcellular location, or both. At nuclear cycle 14, Nuf levels are highest during interphase as the cellularization furrows are forming. Thus, maximal pericentriolar levels of Nuf are correlated with metaphase and cellular furrow formation and invagination. Nuf is highly phosphorylated (Rothwell et al., 1998), raising the possibility that its localization and/or levels may be regulated by cell cycle–dependent kinases.

Nuf dynamics are similar to Rab11 dynamics

Further evidence that Nuf is intimately associated with pericentriolar endosomal material comes from live analysis of Nuf dynamics in the early embryo. This analysis reveals a dynamic punctate distribution of Nuf rapidly moving to and

from the centrosome. Dual imaging reveals that these puncta are closely associated with astral microtubules, and disruption of the microtubule network severely disrupts GFP-Nuf distribution and movement (unpublished data). This colocalization and dependency of the microtubule network has also been demonstrated for Rab11 and GFP-Arfo2 (Mammoto et al., 1999; Hickson et al., 2003). In comparison with live fluorescent analysis of GFP-Rab11 in mammalian systems (Sonnichsen et al., 2000), GFP-Nuf shows a similar localization, distribution, and movement pattern. This supports the view that Nuf localizes to the RE and that these images reflect RE dynamics in the *Drosophila* embryo.

Functional interactions between Nuf and Rab11

Our results also demonstrate a mutual dependence of Nuf and Rab11 for their localization to the RE. In *nuf*-derived embryos, the robust Rab11 pericentriolar distribution is completely disrupted (Fig. 4 A). Whether Nuf is specifically disrupting Rab11 localization to the RE or more globally disrupting RE integrity is not known. However, we believe the effect of Nuf is specific to the RE, as Golgi morphology and distribution is normal in *nuf*-derived embryos (unpublished data). The effect of *nuf* mutations on Rab11 localization is consistent with reports demonstrating that overexpression of GFP-Arfo2 alters the organization of Rab11 in mammalian cells (Hickson et al., 2003).

Conversely, Nuf pericentriolar localization fails in embryos with reduced levels of Rab11 (Fig. 4 B). It has been proposed that endosomes are organized into distinct domains defined by combinations of Rab proteins (Zerial and McBride, 2001). These provide a platform for regulatory/effector proteins to create a distinct fusion-competent domain. The proteins are thought to act cooperatively, and loss of one may destabilize the domain. Nuf and Rab11 may be mutually required for the stable formation of such a domain at the RE of the *Drosophila* embryo.

Nuf and Rab11 are both required for actin and membrane recruitment during metaphase furrow formation

Analysis of nuclear and cortical cytoskeletal defects in *nuf*- and *rab11*-derived embryos supports the idea that Nuf and Rab11 are involved in a similar function at the RE. As observed in the *nuf* mutation, embryos with reduced levels of Rab11 disrupt the syncytial nuclear divisions only after the nuclei reach the cortex. This phenotype indicates that Rab11 is involved in a process specific to the cortical divisions such as cytoskeletal rearrangements or furrow formation. Also like *nuf*, *rab11*-derived embryos exhibit fusions between nonsister nuclei, a hallmark of defective furrow formation (Sullivan et al., 1993).

Previous analysis of *nuf*-derived embryos revealed normal actin organization during interphase, but gaps occur in the actin network early in the process of furrow formation (Rothwell et al., 1998). Our analysis of *rab11*-derived embryos revealed an equivalent phenotype with respect to actin; the interphase actin caps form normally, but the actin-based metaphase furrows are disrupted (Fig. 6 A). Previous analysis of actin dynamics in the *nuf*-derived embryos revealed that actin recruitment during the initial stages of furrow formation is compromised (Rothwell et al., 1999). Our fixed analysis of actin defects in *rab11*-derived embryos reveals actin gaps at the initial stages of furrow formation. Therefore, the *rab11* furrow defects are likely the result of defects in the initial recruitment of actin to the furrows.

Although the *nuf* mutation only partially disrupts actin recruitment to the invaginating furrows, it has a much more severe effect on membrane recruitment. We have used the *Drosophila* homologue of the dystrobrevins, Dah, as a marker for furrow membrane (Zhang et al., 1996). Biochemical analysis demonstrated that this protein associates tightly with actin and membrane, suggesting it is involved in linking the cortical cytoskeleton and the plasma membrane (Zhang et al., 2000). Immunofluorescent analysis reveals that it localizes to the plasma membrane and invaginating furrows, as well as vesicles that accumulate at furrow formation sites (Rothwell et al., 1999). These vesicles are often associated with actin, suggesting that they incorporate as a unit into the growing furrow. In *nuf*-derived embryos, there is some localization of Dah at the furrows; however, most remain in vesicles widely dispersed throughout the cortex (Fig. 6 B; Rothwell et al., 1999). The effect of the *rab11* mutation on Dah localization is even more severe. There is no Dah localization at the furrows, and few Dah-containing vesicles are seen throughout the cortex.

nuf and *rab11* mutations disrupt membrane recruitment and actin remodeling during the early stages of furrow formation, supporting the argument that these proteins function in a common process at the RE. Analysis of Rab11 function in *C. elegans* revealed that it also is important for normal furrow progression during cytokinesis (Skop et al., 2001). However, this analysis showed varying degrees of defects during furrow invagination, suggesting a role for Rab11 during either the initial stages or latter stages (or both) of cytokinesis. In the *Drosophila* embryo, Rab11 appears to be involved in the initial stages of furrow formation when actin is being recruited to the invaginating furrow.

A model for Nuf and Rab11 action at the RE

These analyses indicate that activities of Nuf and Rab11 at the RE influence cortical actin dynamics. Specifically, they direct the recruitment of actin to the sites of metaphase furrow formation. One explanation for this linkage between the endosome and cortical actin dynamics is that membrane and actin are recruited as a unit to the metaphase furrows (Rothwell et al., 1999). Immunofluorescent analysis reveals that Dah-containing vesicles are often tightly associated with actin at the leading edge of the invaginating furrows. Therefore, disrupting membrane recruitment would also disrupt actin recruitment (Fig. 7).

An intriguing alternative explanation for trafficking activities at the RE influencing actin recruitment during the initial stages of furrow formation comes from reports that Rac GTPases are positioned in the cell through the endosomal recycling pathway. For example, Arf6 GTPase regulates an endosomal recycling pathway and cortical actin remodeling at the plasma membrane (Song et al., 1998; Donaldson, 2002). In HeLa cells, ARF6 and Rac1, a potent actin organizer, colocalize at the plasma membrane as well as the RE

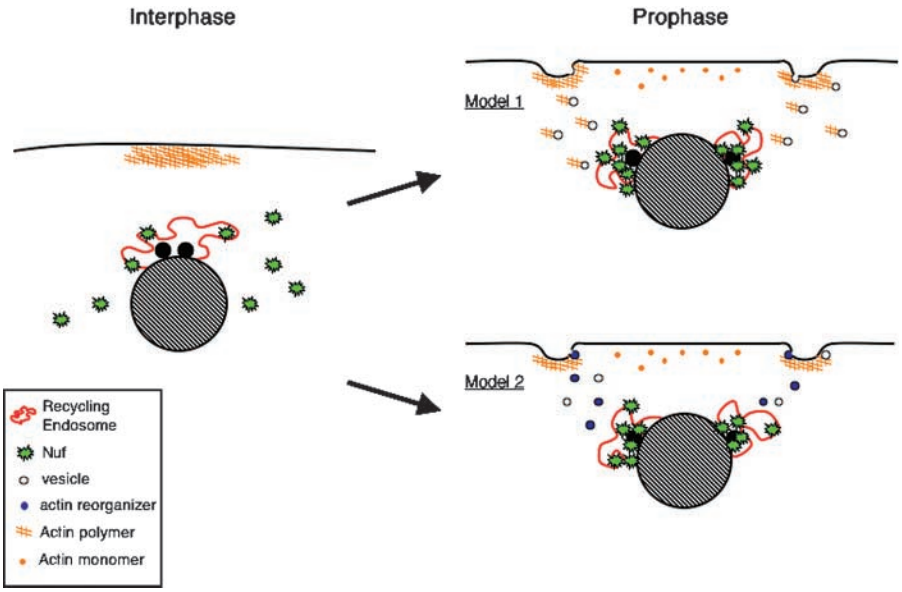

Figure 7. **Nuf links membrane recruitment to actin remodeling during furrow invagination.** Model 1: Nuf localization to the RE during prophase may stimulate vesicle delivery to sites of metaphase furrow formation. Previous reports demonstrated these vesicles are often associated with actin particles, thus membrane and actin are delivered as a unit to the furrows (Rothwell et al., 1999). Model 2: vesicles delivered to the furrow site include potent actin-remodeling factors. This model is based on reports demonstrating that Rac1 is delivered to the membrane through vesicles (Radhakrishna et al., 1999).

(Radhakrishna et al., 1999). Mutational analysis and drug analyses indicate that ARF6 influences actin dynamics by regulating the trafficking of Rac1 to the plasma membrane (Fig. 7; Radhakrishna et al., 1996). This latter model readily explains the effects of Rab11 and Nuf mutations on both actin recruitment and membrane delivery. These proteins are not only required at the RE for membrane delivery to the metaphase and cellularization furrows, but they are also required for the delivery of actin-remodeling proteins, such as Rac, to the plasma membrane.

Cortical actin remodeling and localized plasma membrane expansion not only mediates cytokinetic furrow formation, but also is involved in cell motility, lamellipodia formation, and phagocytosis (Bretscher, 1996; Mellman, 2000). Phagocytosis is particularly interesting because recent work has shown that it occurs through targeted delivery of vesicles from the RE. Accumulation of RE-derived VAMP3-containing vesicles occurs at the site of phagosome formation, and disruption of VAMP3 with tetanus toxin prevents phagosome formation (Hackam et al., 1998; Bajno et al., 2000). As we have demonstrated for metaphase and cellular furrow formation, activity at the RE may also mediate cortical actin cytoskeletal remodeling during phagocytosis.

Materials and methods

Creation of the GFP-Nuf line

A fly strain expressing GFP-Nuf was created by ligating the *nuf* cDNA (bases 318–2274) to the EcoR1 site in the pEGFP-C3 vector, such that Nuf is expressed in frame and downstream of the GFP. Because the EcoR1 site is 15 bases downstream of the start site, the initial five amino acids are absent from the Nuf protein. This construct was inserted into the Nhe1 and Not1 sites in the Germ-10 vector (Serano et al., 1994), provided by Robert Cohen. The Germ-10 vector contains a nurse cell promoter to drive expression in the oocyte. This construct was then used for P-element–mediated transformation (Spradling and Rubin, 1982).

Drosophila stocks

The initial characterization of the *nuf* mutation has been described previously (Sullivan et al., 1993; Rothwell et al., 1998). Oregon-R served as the wild-type control stock (Lindsley and Zimm, 1992). All of the experiments described in this manuscript used the null allele of *nuf* (*nuf*^{1}; Sullivan et al., 1993; Rothwell et al., 1998). *rab11*-deficient embryos were obtained from transheterozygous females bearing the J2D1/93Bi alleles of Rab11 (Jankovics et al., 2001). The J2D1/TM3, Sb and 93Bi/TM3, and Sb stocks were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN). Stocks were maintained on standard maize meal/molasses medium.

Fixation and immunofluorescence

Immunofluorescence analysis was performed as described by Rothwell and Sullivan (2000) and Sisson et al. (2000). Propidium iodide was used to view the DNA. Immunofluorescence analyses using rat anti-Rab11 (supplied by Robert Cohen; Dollar et al., 2002), polyclonal rabbit anti-Nuf (Rothwell et al., 1998), polyclonal rabbit anti-Cnn (supplied by Thomas Kaufman; Indiana University, Bloomington, IN; Megraw et al., 1999), and anti-Dah (Zhang et al., 1996) antibodies were performed on formaldehyde-fixed hand-devitellinized embryos, as described above. Secondary anti–rabbit antibodies, tagged with Cy-5 (Molecular Probes, Inc.), Alexa Fluor® 488 anti–rat (Molecular Probes, Inc.), and Alexa Fluor® 594 anti–mouse (Molecular Probes, Inc.) were applied to the embryos as described previously (Karr and Alberts, 1986).

Live embryo analysis

GFP-Nuf embryos were prepared for microinjection and time-lapse scanning confocal microscopy according to Tram et al. (2001). Rhodamine-conjugated tubulin (Molecular Probes, Inc.) was injected at 50% egg length to view microtubule structures.

Microscopy

Microscopy was performed using an inverted photoscope (DMIRB; Leitz) equipped with a laser confocal imaging system (TCS NT; Leica) and an inverted spinning disk confocal microscope (Eclipse TE200; Nikon). UltraVIEW confocal system CSU10 software (PerkinElmer) was used for the image processing (Wojcik et al., 2001).

Cell culture, transfection, and pull-down assays

Pull-down assays using CHO cells expressing GFP-tagged Nuf and GST-Rab fusion proteins were performed as described by Hickson et al. (2003). The GST-Rab5 vector was provided by Francis Barr.

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