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Integrin Trafficking Regulated by Rab21 Is Necessary for Cytokinesis

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SUMMARY

Adherent cells undergo remarkable changes in shape during cell division. However, the functional interplay between cell adhesion turnover and the mitotic machinery is poorly understood. The endo/exocytic trafficking of integrins is regulated by the small GTPase Rab21, which associates with several integrin α subunits. Here, we show that targeted trafficking of integrins to and from the cleavage furrow is required for successful cytokinesis, and that this is regulated by Rab21. Rab21 activity, integrin-Rab21 association, and integrin endocytosis are all necessary for normal cytokinesis, which becomes impaired when integrin-mediated adhesion at the cleavage furrow fails. We also describe a chromosomal deletion and loss of Rab21 gene expression in human cancer, which leads to the accumulation of multinucleate cells. Importantly, reintroduction of Rab21 rescued this phenotype. In conclusion, Rab21-regulated integrin trafficking is essential for normal cell division, and its defects may contribute to multinucleation and genomic instability, which are hallmarks of cancer.

INTRODUCTION

Cytokinesis is the cell-division process at the end of mitosis in which the membrane is physically cleaved and the daughter cells are separated after chromosome segregation. Although a molecular outline of the sequential events involving alterations in the microtubule cytoskeleton, the formation of an actomyosin contractile ring, and membrane trafficking are known, many aspects of cytokinesis remain poorly characterized (Glotzer, 2005). Nevertheless, understanding cytokinesis is not only important in cell biology, but also in human diseases. A long-standing hypothesis in tumorigenesis is that failures in cell multiplication generate genetically unstable tetraploid cells, which can develop into aneuploid cells with malignant properties (Boveri, 1914; Storchova and Pellman, 2004). It has been demonstrated experimentally that chemically induced tetraploidy (Fujisawa et al., 2005) or random chromosomal missegregations (Weaver et al., 2007) leading to aneuploidy can promote tumor development.

Dividing adherent cells undergo notable changes in shape that are driven by the actin cytoskeleton and microtubule network. Targeted membrane trafficking is also needed throughout the process (Albertson et al., 2005; Boucrot and Kirchhausen, 2007; Konopka et al., 2006; Matheson et al., 2005; Strickland and Burgess, 2004). These same biological entities also participate in the regulation of integrin-dependent cell migration (Pellinen and Ivaska, 2006; Ridley et al., 2003). Nonmotile interphase cells display large matrix adhesion sites called focal adhesions (Geiger et al., 2001), which are disassembled during cell migration or upon initiation of mitosis with concomitant changes in cell morphology (Ezratty et al., 2005; Maddox and Burridge, 2003). Adherent cells held in suspension fail to undergo cytokinesis, resulting in binucleation (Ben-Ze’ev and Raz, 1981; Kanada et al., 2005; Pugacheva et al., 2006; Thullberg et al., 2007). In addition, β1-integrin expression is required for cytokinesis in chondrocytes in vivo (Aszodi et al., 2003). These findings suggest that integrin-mediated adhesion may be necessary for successful mitosis. However, the mechanisms involved are not known.

Many integrins are constantly endocytosed and recycled to the plasma membrane (Pellinen and Ivaska, 2006), facilitating targeting of these adhesion receptors during cell migration (Bretscher, 1989). Integrin trafficking requires the spatially regulated activity of a number of kinases in conjunction with Rab-dependent endosomal compartments (Caswell and Norman, 2006). The ubiquitously expressed, small GTPase Rab21 has recently been shown to associate with the α tails of several integrins via the shared conserved membrane proximal sequence, thus regulating cell adhesion and migration via controlling the endo/exocytic trafficking of most integrin heterodimers (Pellinen et al., 2006). More recently, association between Rab25 and the integrin β1 subunit has been shown to regulate integrin trafficking and invasion by a mechanism restricted to epithelial cells cultured in three-dimensional matrix (Caswell et al., 2007). Whereas the role of integrin trafficking during migration is recognized, almost nothing is known about the role of integrin targeting in cell division.

Since errors in cell division can contribute to aneuploidy, which is a critical early step in cancer development (Piekny et al., 2005), we have investigated integrin turnover during cell division.
division in detail. Given the analogy between changes in cell morphology during migration and mitosis, we hypothesized that Rab21 could function to support both processes. We demonstrate here that the targeted trafficking of integrins from the cell surface to the cytosolic compartment is necessary for cytokinesis in different cell types and organisms. Mechanistically, we show that Rab21 is required for integrin trafficking to the cleavage furrow of dividing cells. We also demonstrate that human cancer cells can harbor Rab21 deletions, causing impaired cytokinesis and multinucleation. These studies therefore reveal how the coordinated trafficking of integrins is necessary for cell division and link this process to human cancer.

RESULTS

β1-Integrins Anchor the Cleavage Furrow to Matrix in Cytokinesis

To gain insight into the function and turnover of integrins during cell division, we investigated the localization of integrins during mitosis (Figures 1A–1E). In prometaphase and metaphase cells, β1-integrin localized diffusely at the membrane and in a few intracellular vesicles (Figures 1A and 1B). In early telophase, integrin was detected mainly on the matrix-facing side of the cells (Figure 1C, z axis, arrowhead), and by mid-telophase, β1-integrin and actin overlap in the ingressing cleavage furrow (arrow), and β1-integrin shows prominent localization at the basal side of the cleavage furrow (Figure 1D, arrowhead). Strong overlap of β1-integrin and actin as well as enrichment of β1-integrin at the basal side of the furrow were detected in 66% of the cells investigated (n = 32). In late telophase, the intercellular bridge was no longer connected to the substrate, and β1-integrin was concentrated to the extending protrusions formed at the opposite poles of the daughter cells (Figure 1E, arrow).

The close proximity of the cleavage furrow to the underlying matrix suggested that integrin-mediated adhesion or integrin signaling may play a functional role in cytokinesis. To investigate this, we analyzed cytokinesis in live cells treated with function-blocking β1-integrin antibody (Wayner et al., 1993). The integrin-antibody complex was internalized and moved to the furrow (data not shown). However, the inability of the accumulated integrins to adhere to matrix resulted in an increased number of cells arrested in cytokinesis compared to control cells (Figure 1F, arrows; Movie S1 available online). This suggests that integrins accumulating to the cleavage furrow function either to anchor the structure or to trigger matrix-induced signaling and thus support the execution of cytokinesis.

Perturbation of the Rab21 GTPase Cycle Blocks Cytokinesis

We have shown earlier that Rab21 small GTPase associates with integrin cargo and targets them to the endocytic machinery in migrating cells (Pellinen et al., 2006). Since integrin trafficking could also regulate cell division, we examined whether perturbation of Rab21 activity affects cell division. Rab21 function can be inhibited by the expression of mutant Rab21 locked in the GTP form (Pellinen et al., 2006). Expression of Rab21GTP induced a bi- or multinucleate phenotype in different cell types derived from various species, including human lung cancer cells (NCI-H460), mouse embryonic fibroblasts (MEFs), and Chinese hamster ovary (CHO) cells (Figure 2A; Figures 2B and 2C).
Figures S1A and S4A). A total of 16 hr after transfection, 69% ± 5% of GFP-Rab21GTP-expressing NCI-H460 cells were bi- or multinucleate, compared to 11% ± 3% of nontransfected cells (Figure 2B). Treatment of Rab21GTP-expressing cells with the microtubule-depolymerizing drug nocodazole led to pseudo metaphase arrest, indicating that mitotic checkpoint signaling was not compromised in these cells (Figure 2B; Figure S1A).

To investigate whether the observed bi- and multinucleation was due to defects in cytokinesis, we examined Rab21GTP-expressing cells by time-lapse microscopy. Nontransfected and GFP-Rab21-expressing NCI-H460 cells completed mitosis in 51 ± 8 min (n = 5 cells) after cell rounding upon entry into M phase (Figure 2C; Movie S2), whereas Rab21GTP-expressing cells made futile attempts to complete cytokinesis and fuse to form a binucleate cell. The numbers in the frames indicate minutes. The first panels show GFP expression.

Figure 2. Rab21 Regulates Cytokinesis
(A) GFP-Rab21 induces bi- and multinucleation. GFP-Rab21GTP-transfected NCI-H460 lung cancer cells (indicated with asterisks), stained as indicated, show an increased number of multinucleate cells (arrows) compared to GFP-Rab21-expressing cells. The scale bar represents 10 μm. (B) Rab21GTP induces multinucleation downstream of the mitotic checkpoint. NCI-H460 cells transfected as indicated were scored 8 or 16 hr posttransfection in the presence or absence of 1 μM nocodazole for mono- and multinuclear cells (mean ± SEM, n = 54–138 per group; ***p < 0.001). (C) GFP-Rab21GTP induces a failure in cytokinesis. Representative still images of time-lapse analysis of NCI-H460 cells transfected as indicated undergoing cytokinesis. A Rab21-expressing cell completes cytokinesis (top row), whereas a Rab21GTP-expressing cell fails to complete cytokinesis and fuses to form a binucleate cell. The numbers in the frames indicate minutes. The first panels show GFP expression. (D) Expression of GFP-Rab21GTP does not influence Aurora B localization during metaphase and anaphase, but disables the normal accumulation to the mid-body. NCI-H460 cells were transfected and stained as indicated. The scale bar represents 10 μm.
Integrin Trafficking Regulates Cytokinesis

Integrin Targeting to the Cleavage Furrow Is Rab21 Dependent

To investigate the specific role of Rab21 in the process, we silenced Rab21 with siRNA that has previously been shown to be specific for Rab21, to inhibit migration identically to another Rab21-targeting siRNA oligo, and to not influence the expression levels of integrins, other endosomal Rabs, or EEA1 (Pellinen et al., 2006). Importantly, partial silencing of Rab21 with siRNA (~65%), which inhibits integrin trafficking (Pellinen et al., 2006), also significantly increased the number of multinucleated cells (by 15% ± 4%) compared to control siRNA-transfected cells (Figure 3A). These findings with siRNA silencing indicate a specific role for Rab21 during cytokinesis.

Since Rab21 regulates the endo/exocytic trafficking of β1-integrins, and since active integrins localize to endocytic Rab21 vesicles in migrating cells (Pellinen et al., 2006), we investigated whether Rab21 regulates the localization of β1-integrins during mitosis. In mid-telophase, endogenous Rab21 and β1-integrin were found to overlap in the ingressing cleavage furrow (Figure 3B, arrows; Figure 3C, left panels, arrow), and β1-integrins were again detected at the base of the furrow adjacent to the matrix (Figure 3B, z axis, arrowhead). Silencing of Rab21 expression prevented concentration of β1-integrin at the furrow (Figure 3C, right panels), suggesting a role for Rab21 in the recruitment of the integrins to the furrow. In late telophase, β1-integrin and Rab21 were detected at the opposite poles of the daughter cells. Their localization overlapped in vesicles at the base of the integrin-containing lamellipodia formed by the separating daughter cells (Figure 3B, arrows). Newly formed protrusions containing β1-integrin were visible at the level of the matrix, suggesting that these ruffles are engaging matrix to assist mechanically in the separation of the two daughter cells (Figure 3B, right panel, z axis, arrowheads).

These data suggest that integrins play a mechanistic adhesive role during telophase. However, it is well established that integrin-matrix interactions generate intracellular signals regulating several pathways, including the activity of RhoA (Ren et al., 1999). Active RhoA forms a narrow zone at the site of the forming contractile ring and is essential for furrowing and cytokinesis (Bement et al., 2005; Yuce et al., 2005). To evaluate whether accumulation of β1-integrin at the furrow correlates with the localization of active RhoA in the furrow, we used an affinity probe, based on the GFP-fused Rho-binding domain (RBD) of Rhotekin, which only binds to Rho in its active conformation (Berdexola et al., 2004). In the control cells, this probe bound preferably to a narrow zone in the cleavage furrow during telophase (Figure 3D). The GFP-only probe was not detected in the furrow (Figure 3D, bottom panels). In contrast, in Rab21-silenced cells, the GFP-RBD probe accumulated to the furrow in only 22% ± 6% of the cells, compared to the 91% ± 4% of positive furrows in the control cells (p = 0.001; n = 30 cells per group) (Figure 3D). In summary, Rab21 regulates the accumulation of integrins into the cleavage furrow, and integrin accumulation correlates with the localization of active RhoA in the furrow. Therefore, it is possible that integrin signaling in the furrow may play a role in regulating RhoA activity in conjunction with known RhoA effectors (Yuce et al., 2005; Zhao and Fang, 2005).

β1-integrins can traffic through a Rab11-positive compartment (Powelka et al., 2004; Roberts et al., 2001) and the...
Figure 4. Rab21-Regulated Trafficking of Integrins Is Necessary for Cytokinesis

(A) Schematic representation of the integrin α2 subunit cytoplasmic domain mutations at the indicated residues (green) in the conserved region shared by all α subunits (red box).

(B) Mutant α2-integrins, unable to associate with Rab21, fail to support cell division on collagen. The indicated α2-integrin constructs were expressed in CHO cells, and cell division was investigated in CD hybridoma medium on collagen (exogenous α2β1-mediated adhesion) or fibronectin (endogenous α5β1-mediated adhesion). The scale bar represents 10 μm.

(C) Mutant α2AA- and α2P-integrins are bi- or multinucleate on collagen (arrows, mean ± SEM, n = 32–79 cells per group; ***p < 0.001).

(D) α2AAβ1-integrin is not endocytosed. Integrins were immunoprecipitated from surface-biotinylated cells by using anti-α2 antibody, and the amount internalized was determined by bloting for biotin (relative to total precipitated α2-integrin; mean ± SEM, n = 3).

Developmental Cell
Integrin Trafficking Regulates Cytokinesis

Rab11-binding protein FIP3 has emerged as a regulator of endosome transport to the cleavage furrow during cytokinesis (Fielding et al., 2005; Simon et al., 2008; Wilson et al., 2005). We investigated the role of FIP3 in the regulation of integrin localization during cytokinesis by silencing FIP3. Transfection with a previously described FIP3-specific siRNA oligo (Wilson et al., 2005) reduced FIP3 mRNA levels by 97% ± 2% relative to control siRNA-transfected cells (qRT-PCR, n = 3, p = 0.01). We also observed that FIP3-silenced cells were unable to complete cytokinesis, but arrested in mid/late telophase. In contrast to Rab21-silenced cells, β1-integrin accumulated to the furrow during telophase in FIP3-silenced and control cells (Figure S3A, arrow). However, in line with the role of FIP3 in Rab11-dependent recycling, FIP3-silenced cells displayed accumulation of β1-integrin in the perinuclear region that was not detected in control or Rab21-silenced cells (Figure S3B; data not shown). Silencing of FIP3 also induced abnormal actin filaments at the bottom of the dividing cells without significantly interfering with actin localization at the furrow (Figure S3C). Interestingly, simultaneous silencing of FIP3 and overexpression of GFP-Rab21 interfered with the ability of NCI-H460 cells to enter mitosis (0 out of more than 400 cells scored were in mitosis). Furthermore, silencing of FIP3 has dramatic effects on GFP-Rab21 (Figure S3D). GFP-Rab21 is localized to human intracellular vacuoles in addition to smaller motile vesicles, possibly reflecting the cumulative effects of simultaneously enhancing endocytosis (Rab21) and inhibiting recycling (FIP3). Taken together, these results suggest that both Rab21 and FIP3 are important in regulating integrin localization in dividing cells, but that their functions are not fully overlapping, and that integrin accumulation to the cleavage furrow occurs in the absence of FIP3.

Since our results imply that cell division may involve the directional movement of integrins and Rab21, we investigated the localization of Rab21 and α5-integrin in live cells. Indeed, GFP-α5-integrin andDsRedm-Rab21 overlapped in late mitotic cells in vesicles accumulating first to the vicinity of the ingressing cleavage furrow, and later in telophase to the opposite ends of the two spreading daughter cells (Figure 3E; Movie S4). This was investigated further by tracking the movement of antibody-labeled integrins at the furrow. Analysis of the trajectories of individual intracellular integrin vesicles on average, 103 vesicles per active furrow region [166 µm²] demonstrated that, during early telophase, integrins traffic toward the furrow (Figure 3F; Table S1; Movie S5). In contrast, in Rab21-silenced cells, we detected a smaller amount of endocytosed-labeled integrin (on average, 68 vesicles per active furrow region), and the detected integrin vesicles were more stationary (Table S1). The directional movement toward the furrow was impaired in Rab21-silenced cells (Figure 3F; Table S1; Movie S6), and some vesicles were moving in the opposite direction (Figure 3F, siRab21, light-green and dark-green tracks). Interestingly, the silencing of FIP3 did not influence the number of integrin vesicles in the furrow significantly (on average, 95 vesicles per active furrow region), further supporting our finding that integrins accumulate to the furrow in FIP3-silenced cells during cytokinesis (Figure S3A). In late cytokinesis, trajectories revealed that in control cells integrins were trafficking away from the mid-body toward the opposite end of the forming daughter cells (Figure 3F; Table S1; Movie S7); this movement was also impaired in Rab21-silenced cells (Figure 3F; Table S1; Movie S8).

Taken together, Rab21 and integrin have overlapping localization in dividing cells. Loss of Rab21 function results in impaired integrin localization to the cleavage furrow and defective cytokinesis, leading to bi- and multinucleated cells.

**Rab21 Association with Integrin Is Necessary for Cytokinesis**

Rab21 regulates the trafficking of all α/β1-integrin heterodimers (including collagen-binding α2β1-integrin) by associating with the conserved membrane-proximal GFFKR sequence present in almost all integrin α subunits (Hynes, 2002). Thus, by interfering with integrin-Rab21 association via mutagenesis of an α subunit that pairs with the β1 subunit, we can specifically address the requirement of Rab21-regulated integrin trafficking in supporting cytokinesis. CHO cells fail to express collagen-binding integrins (Nykvist et al., 2000) and thus can be reconstituted with mutant α2-integrins and subsequently investigated on collagen substrates. We found that CHO cells become bi- or multinucleate when they express Rab21GTP (Figure S4A). Substitution of the highly conserved arginine (R1161) with an alanine residue (α2AA mutant) or disruption of the α-helical structure of the α2 subunit’s cytoplasmic tail by exchanging the lysine residue (K1162) with a proline (α2P mutant) abolished the association of Rab21 with the α2 (Figure 4A) (Pellinen et al., 2006). Introduction of wild-type integrin α2 subunit rendered CHO cells able to adhere and divide on collagen as efficiently as on fibronectin, to which they adhere via their endogenous α5β1 (Figure 2B). Strikingly, cells expressing mutant α2-integrins unable to recruit Rab21 (α2AA and α2P) adhered to collagen, but showed a bi-/multinucleate phenotype indicative of defective cytokinesis, whereas, on fibronectin, the same cells appeared to undergo normal cytokinesis (Figure 4B). These populations contained 64% ± 9% (α2AA) and 78% ± 12% (α2P) bi- or multinucleate cells when cultured on collagen (Figure 4C) and 2% ± 1% (α2AA) and 4% ± 1% (α2P) bi- or multinucleate cells when grown on fibronectin. The α2ARA mutant, which retains the ability to associate with Rab21 (Figure 4A) (Pellinen et al., 2006), supported normal cell division on collagen as well as on fibronectin (Figures 4B and 4C). These data suggest that the association of Rab21 with the conserved GFFKR sequence found in most integrin α subunits is necessary for cytokinesis.

Next, we examined the trafficking of α2β1-integrin by determining the internalized and cell surface receptor-bound integrin fractions (Roberts et al., 2001). As expected, the mutant α2AA-integrin, which cannot associate with Rab21, failed to undergo endo/exocytic trafficking, whereas α2ARA was efficiently internalized from the cell surface (Figure 4D). In line with these data, in individual confocal slices taken from the middle of the cell, α2ARA was in vesicles in the cytoplasm and at the cleavage furrow (56 ± 8 vesicles per α2ARA’s active furrow region) in...
addition to the membrane. In contrast, the α2αA mutant was detected as being evenly distributed along the plasma membrane and only in very few vesicles close to the furrow (6 ± 3 vesicles per α2αA’s active furrow region) (Figure 4E). Taken together, the loss of integrin trafficking either due to the expression of the Rab21GTP mutant or defective integrin-Rab21 association causes mitotic errors, resulting in multinucleate progeny cells.

**Mutagenesis of Conserved β1-Integrin NXXY Motifs Blocks Endocytosis and Cytokinesis**

The exact pathways involved in the endocytosis of integrins are not clearly defined and seem to vary between integrin heterodimers (Ramsay et al., 2007; Upla et al., 2004). However, recent reports have implicated clathrin-coated structures in the endocytosis of integrins (Nishimura and Kaibuchi, 2007; Ramsay et al., 2007). The cytoplasmic domains of β-integrins contain two conserved NXXY motifs required in other cell surface receptors for clathrin-mediated endocytosis (LeRoy and Wrana, 2005), suggesting that they also might be involved in the regulation of integrin trafficking.

We therefore investigated the endocytic trafficking in β1-integrin null MEFs expressing either wild-type β1-integrins or β1-integrins in which the tyrosine residues of the cytoplasmic domain were substituted with phenyalanines ([β1YY783,795FF] (Wennerberg et al., 2000). Wild-type β1-integrin expressed in GD25 cells localized to intracellular structures and focal adhesions (Figure 5A, arrowheads and arrows), whereas [β1YY783,795FF-integrins localized to prominent focal adhesions, typical of cells with inhibited focal adhesion turnover (Ezratty et al., 2005). Furthermore, wild-type β1-integrin was rapidly endocytosed from the cell surface (Figure 5A). In contrast, [β1YFF-integrin showed reduced endocytosis (Figure 5A). This was not due to an overall trafficking defect since the endocytosis of labeled transferrin was unaltered in cells expressing the mutant integrins (Figure S4B). The GD25 cells are derived from differentiated β1-integrin-deficient embryonic stem cells (Fassler and Meyer, 1995) and therefore could have some specific features not observed in normally differentiated cells. To further verify the role of β1-integrin NXXY motifs in integrin endocytosis, we investigated integrin endocytosis in β1 wild-type and β1YYFF MEFs isolated and cloned from E13.5 embryos, which carry the mutation in the germline (Czuchra et al., 2006). Also, in these cell clones derived from the β1YFF embryos, integrin endocytosis was reduced compared to cells derived from wild-type β1-integrin littermates (Figure 5B).

Since clathrin-mediated endocytosis is important for cytokinesis (Figure S4C (Niswonger and O’Halloran, 1997), we examined cytokinesis of the embryo-derived wild-type and β1YYFF cells on selective matrices. Laminin is only recognized by β1-integrins, whereas adhesion to vitronectin is mainly mediated via β3-integrins (Hynes, 2002). We exploited this specificity to investigate whether endocytosis-deficient β1YYFF MEFs are able to divide on laminin. We found that β1YYFF cells cultured on vitronectin were mainly mononuclear, whereas the same cells on laminin were significantly more frequently bi- and multinucleate (Figures 5C and 5D, arrowheads). In accordance with this finding, ES cell-derived GD25 β1 null cells reconstituted with wild-type β1-integrin appeared normal on laminin and vitronectin. In contrast, GD25 cells reconstituted with β1YYFF became bi- or multinucleate, specifically on laminin, where adhesion is mediated via the mutant integrin alone (Figure 5D).

Overexpression of Rab21 stimulates integrin trafficking and increases cell migration on β1-integrin substrates (Pellinen et al., 2006). To analyze whether the defective cell division of β1YYFF cells on laminin is due to impaired integrin trafficking, we tested whether overexpression of Rab21 could rescue the multinucleate phenotype of the GD25 β1YYFF cells. Overexpression of Rab21, but not of Rab7, which does not associate with β1-integrins (Pellinen et al., 2006), indeed rescued the multinucleated phenotype (Figure 6A; Figure S4D) and integrin endocytosis (Figure 6B) in GD25 β1YYFF cells. Interestingly, whereas endocytosis of wild-type β1-integrin was sensitive to inhibition of clathrin by mono-dansyl-cadaverin (MDC) (Davies et al., 1980), the Rab21-induced endocytosis of β1YYFF-integrin was MDC insensitive (Figures S5A and S5B). Thus, these data suggest that in the context of the mutant β1-integrin Rab21 expression induces integrin trafficking by stimulating some form of clathrin-independent endocytosis.

Previous work has demonstrated that, in the absence of functional β1-integrin, MEFs upregulate the expression of β3-integrin (Danen et al., 2002). This was also the case in the β1YYFF-expressing cells. Surface expression of endogenous β3-integrin was low in GD25 β1 cells (mean fluorescence intensity [MFI] = 105), whereas in GD25 β1YYFF cells expression of β3 was upregulated (MFI = 1253) similarly to what has been demonstrated for β1 null cells (Retta et al., 2001). Interestingly, we observed that in GD25 β1 cells β1-integrin, but not β3-integrin, accumulated in the cleavage furrow. In contrast, in GD25 β1YYFF cells, the upregulated β3 instead of the mutant β1 was recruited to the furrow (Figure 6C). Therefore, our data suggest that β3-integrin is upregulated to compensate for the previously unknown inability of mutant β1-integrin to undergo trafficking and thus support cytokinesis.

Finally, we tested the ability of Rab21 to rescue cytokinesis in the context of an integrin with mutations in Rab21 association. We expressed Rab21 together with α2wt- or α2αA-integrins in CHO cells cultured on collagen. Rab21-expressing α2αA cells remained bi- or multinucleate on collagen (Figure 6D, arrows; Figure S4E), demonstrating that Rab21 facilitates cytokinesis specifically via its ability to associate with integrins and stimulate their endocytosis.

**Cancer Cells Harboring a Rab21 Deletion Are Multinucleate**

A recent report suggests that tetraploid cells, forming as a result of cytokinesis failure, have an increased number of chromosome rearrangements, directly facilitating malignant transformation and tumorigenesis (Fujivara et al., 2005). Since our data strongly suggest that Rab21 is necessary for normal cell division, and that impaired Rab21 function results in multiploidy, we investigated the Rab21 gene in human cancer. We performed array CGH analysis of two ovarian carcinoma cell lines (parental KF28 and variant KFr13) and found a deletion in the Rab21 locus on chromosome 12 in the KFr13 cells (Figure 7A). The deletion resulted in a significant loss of Rab21 mRNA and protein expression when compared to the parental KF28 cells with an intact Rab21 gene locus (Figure 7B). In contrast, protein levels of β1-integrin and
Rab5 were identical in KF28 and KFr13 cells (data not shown). Interestingly, a similar chromosomal deletion involving the RAB21 locus and also leading to loss of RAB21 expression was seen in a prostate cancer tumor sample (Figure S6; data not shown).

We investigated whether loss of the Rab21 gene altered the phenotype of these cells. Freshly plated Rab21-negative KFr13 cells displayed increased accumulation of multinucleate cells (after 24 hr, 16% ± 6%, n = 100; p = 0.0003 two-sample test for equality) (Figure 7C, arrows), when compared to Rab21-expressing KF28 cells (0%; n = 100). Importantly, these results correlate well with the phenotype of Rab21-silenced NCI-H460 cells (Figure 3A). Reintroduction of GFP-Rab21, but not GFP...
alone, maintained mononuclear KFr13 cells (Figure 7D). By contrast, expression of the GFP-Rab21GTP mutant resulted in a further increase in the multinucleate phenotype (Figure 7D). Furthermore, silencing of Rab21 in KF28 cells phenocopied the multinucleate phenotype of the KFr13 cells (Figure 7E). Thus, RAB21 inactivation caused by acquired genetic changes in cancer could be functionally linked to multinucleation in a way analogous to our experimental models. This suggests that dysregulation of integrin trafficking by mechanisms such as Rab21 inactivation may give rise to multinucleation and contribute to genetic instability and tumor progression.

**DISCUSSION**

We have demonstrated that Rab21 controls the endosomal trafficking of integrins, which is required for the successful completion of cytokinesis. These results provide mechanistic insight into the over 2-decade-old observations that matrix adhesion is a prerequisite for the division of adherent cells (Ben-Ze’ev and Raz, 1981; Orly and Sato, 1979). We further demonstrate that the RAB21 gene locus can be deleted in cancer, resulting in multinucleate cells. This provides one example of how genetic instability may arise from cellular multinucleation due to impaired...
integrin trafficking in human cancer, thus linking our findings to a far-older hypothesis suggesting that aneuploidy may drive tumorigenesis (Boveri, 1914). Both aspects have important implications for human disease.

Anchorage-independent growth is a hallmark of cancer. However, the mechanism governing the anchorage dependence of cell division has been unclear. We demonstrate here that integrins accumulate to the cleavage furrow and function to anchor...
the ingressing furrow to the matrix (Figure 1). Integrins may have several functions in the cleavage furrow. Our experiments demonstrate that integrin accumulation to the furrow must be able to engage the matrix to support cytokinesis. Therefore, it is likely that one important function of integrins in the furrow is to provide mechanical anchoring for the contractile actomyosin ring. The contractile ring assembly is directed by Rab21 via activation of myosin and induction of actin nucleation (Glötzler, 2005). Integrins are known regulators of Rab21 activity (Hall, 2005), and thus integrins directed to the furrow might contribute to locally regulate Rab21 activity. This possibility is supported by our data showing that, in the absence of functional Rab21, localization of active Rab21 to the furrow is impaired. The integrin β subunit may also be necessary to recruit focal adhesion proteins like α-actinin and talin to the cleavage furrow (Bellissent-Waydelich et al., 1999; Fujiwara et al., 1978). Integrins are known to assemble complex signaling modules at sites of adhesion (Zaidel-Bar et al., 2007), and the assembly of similar complexes governing cytokinesis-specific signaling molecules may exist. Therefore, it is reasonable to assume that integrins are required in the temporal assembly and that anchoring of the contractile ring is needed for normal cytokinesis.

In this study, we have investigated the role of integrins and their trafficking in cells undergoing cytokinesis. Our results demonstrate that, first, β1-integrins traffic in a spatiotemporally controlled manner to the ingressing cleavage furrow and later to the opposing poles of the daughter cells during cytokinesis (a schematic representation is shown in Figure S7). Second, Rab21 association with the conserved GFFKR sequence in the integrin α subunit regulates trafficking of integrins during cytokinesis. Third, clathrin- and Rab21-dependent endocytosis of the receptor both contribute to integrin trafficking to the furrow. Fourth, Rab21 expression can compensate for the aberration of putative clathrin-binding sites in the integrin β1 tail and rescue trafficking and cytokinesis by directing β1YYFF endocytosis to a non-clathrin-dependent pathway. Our findings that mutant integrins undergoing reduced integrin trafficking fail to support cytokinesis when matrix adhesion is mediated solely by the mutant integrin heterodimer (Figures 4 and 6) are in line with a similar observation made in vivo. Loss of β1-integrin expression results in a cytokinesis defect in chondrocytes that adhere via different members of the β1-integrin subfamily (α1β1, α2β1, α10β1, and α11β1) to the collagen II matrix in the cartilage (Aszodi et al., 2003). Moreover, β1YYFF mutant keratinocytes, adhering to the complex matrix of the epidermal-dermal basement membrane with several integrin subfamilies, show no obvious defects in vivo (Czuchra et al., 2006), whereas cells isolated from these mice fail to execute cytokinesis on a β1-specific matrix in vitro (Figure 5). Furthermore, a previous study has demonstrated that an inactive mutant integrin is also unable to support cytokinesis (Reverte et al., 2006), further underlining the mechanistic adhesive role of integrins in cytokinesis. Importantly, the mutant β1YYFF-integrin is however fully active (data not shown). Therefore, our findings are, to the best of our knowledge, the first to provide evidence that impaired integrin trafficking may be linked to an abortive cell cycle, and changes in integrin subcellular localization, regulated by Rab21, need to be precisely timed during cell division.

It is well established by many reports that endo/exocytic trafficking of membranes is necessary during cytokinesis. Thus far, membrane trafficking to the furrow has been shown to support the polarized deposition of lipids and proteins to the furrow, and the addition of new membrane to provide the necessary surface is required for furrow ingression (for a review, see [Simon and Prekeris, 2008]). Our data show that in addition to the general requirement for membrane trafficking during cytokinesis, specific trafficking of integrins is important for the process in adherent cells. We show that in addition to Rab21, FIP3 is required for spatiotemporal control of integrin localization during cytokinesis. Our data show that β1-integrins can be delivered to the furrow in the absence of FIP3, but that in FIP3-silenced cells arrested in telophase, a pool of perinuclear β1-integrin persists. It is possible that trafficking of these receptors is necessary for the subsequent separation of daughter cells, which involves the formation of new integrin-positive protrusions.

Tetraploid cells represent genetically unstable intermediates that develop into aneuploid cells due to the inability of these cells to segregate their chromosomes correctly (Barr and Gruneberg, 2007). We show here that the RAB21 gene can be deleted in human cancer (Figure 7; Figure S5), and that this results in multinucleation, which can be rescued by the reintroduction of Rab21. These findings suggest an interesting mechanism for how one genetic alteration in cancer can promote failure in normal cell division and thus result in further genetic instability.

Taken together, these findings reveal a molecular mechanism by which Rab21 governs integrin endosomal trafficking during cytokinesis and raise the possibility that alterations in integrin trafficking may lead to the development of genetic instability as a result of cytokinesis failure. This is likely to be relevant in human malignancies in which impaired mitosis leading to polyploidy has been proposed as a causative effect in the formation of aneuploidy and in the promotion of cancer progression (Storchova and Pellman, 2004).

**EXPERIMENTAL PROCEDURES**

Details of DNA constructs, siRNA sequences, antibodies, and the generation of mouse embryonic fibroblasts (MEFs) from β1wt and β1YYFF mice (Czuchra et al., 2006) are described in Supplemental Experimental Procedures.

**Cell Culture, DNA Transfection, and RNA Interference**

NCI-H460, Chinese hamster ovary (CHO), and HEK293 cells were obtained from ATCC. GD25 mouse fibroblasts have been described (Wennerberg et al., 2000), and KFr13 and KFr28 cells (Kikuchi et al., 1986) were a generous gift from Dr. N. Sasaki. All DNA constructs were transfected by using Lipofectamine 2000 (Invitrogen). Long-term, siRNA-mediated gene silencing was done with HiPerfect transfection reagent (Qiagen) according to the manufacturer’s protocol. The molar concentrations of siRNAs ranged between 100 and 130 nM in all experiments.

**Cell Proliferation Analysis**

Cell proliferation of siRNA-transfected cells was measured by using WST-1 (Roche) according to the manufacturer’s protocol. NCI-H460 cells were plated on clear-bottom, 96-well plates (COSTAR) and were transfected with RAB21 siRNA or control siRNA. Cell proliferation (absorbance at 450 nm) in wells was measured at time points 0, 24, and 48 hr.

**Microscopy**

For imaging of fixed cells, cells were plated on acid-washed, glass coverslips coated with 5 µg/ml collagen, fibronectin, vitronectin, or laminin. Cells were...
fixed with 3.7% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS for 15 min, and blocked with 2% BSA/PBS for 30 min. Primary antibodies were used with predetermined optimal concentrations of 5–10 μg/ml. The concentration of Alexa-conjugated secondary antibodies (Invitrogen) was 5 μg/ml. GFP-RBD for the detection of active Rho was generated as described in Goulimari et al. (2005). For detection of active Rho, cells were fixed in 3.7% paraformaldehyde, 5 mM EGTA, 1.5 mM MgCl₂ in PBS; permeabilized with 0.5% Triton X-100, 5 mM EGTA, 1.5 mM MgCl₂ in PBS; and incubated with GFP-RBD (0.05 μg/ml) or GFP alone (0.03 μg/ml) in 2% BSA, 5 mM EGTA, 1.5 mM MgCl₂ in PBS. Coverslips were mounted with Vectashield mounting medium (Vector Labs) containing DAPI for nuclear staining. Alexa-conjugated phalloidin (Invitrogen) was used for F-actin detection.

In live-cell imaging of NCI-H460 or GD25/1, cells transfected with EGFP-Rab21GTP or EGFP-Rab21 were imaged starting 8 hr after transfection. Phase-contrast images were taken (EL Plan-Neofluar 20×0.5 NA objective, 6 frames/hr) with a Zeiss inverted wide-field microscope equipped with a heated chamber (37°C) and CO₂ controller (4.8%) for 20–24 hr. GD25/1 cells were imaged in CO hydriodema medium ( Gibco-BRL). Confocal live-cell imaging of α5-GFP and DsRed-Rab21 was done by seeding transfected HEK293 cells onto 60 μ-Dish plates (Ibidi) coated with 10 μg/ml fibronectin. Z-stacks of 8 planes (1 airy unit optical slices, step size of 0.3 μm between slices) were taken every 2 min. Confocal live-cell imaging of endogenous integrin was done by labeling cells directly with mouse α2-integrin antibody (MA20225) together with anti-mouse Alexa 488 secondary antibody. Cells were allowed to internalize antibodies for 1 hr at +37°C together with anti-mouse Alexa 488 secondary antibody. Cells were allowed to do by labeling cells directly with mouse integrin. Confocal three-dimensional images were taken by using Zeiss inverted wide-field microscope (O.D Plan-Neofluar 40×/0.6 NA Korr objective, 6 frames/hr). As controls, cells were treated with the same medium without the P5D2 antibody. After 16 hr of imaging, cells in cytokinesis were scored manually.

Immunofluorescent samples were analyzed with a Zeiss inverted wide-field microscope by using a Zeiss Plan-Neofluar 63× oil/1.4 NA objective complemented with MetaMorph imaging software. Maximum projections of images were created from Z-stacks (step interval of 0.2–2 μm). Confocal three-dimensional images were taken with using Zeiss Axiocam R3 with the spinning disc confocal unit Yokogawa CSU22 and a Zeiss Plan-Neofluar 63× oil/1.4 NA objective. Z-stacks with 1 airy unit optical slices were acquired with a step size of 0.3 μm between slices, and the maximum intensity projections were created with SlideBook 4.2.0.7 software and NIH ImageJ. QuickTime movies from time-lapse experiments were created by using NIH ImageJ software.

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**mization Assays**

Integrin internalization assays were performed as described earlier (Pellinen et al., 2006; Roberts et al., 2001). Internalized integrin was blotted with anti-biotin-HRP, and the total immunoprecipitated integrin was blotted with either anti-α2-integrin or anti-α1-integrin.

**Sample Preparation, Gene Copy Number, and Expression Data**

Genomic DNA and total RNA from KF28 and KF13 cell lines were extracted by using QIAamp and RNeasy mini kits, respectively (QIAGEN, GmbH, Hilden, Germany).

Array-based CGH was performed by using Agilent 44k oligo microarrays according to the protocol (Agilent Technologies, Palo Alto, CA), with minor modifications. Female genomic DNA (Promega, Madison, WI) was used as hybridization controls. Briefly, 3 μg digested and purified tumor and reference DNA were labeled with Cy5-dUTP and Cy3-dUTP (Perkin-Elmer, Wellesley, MA), respectively, in a random priming reaction by using Bioprint Array CGH Genomic Labeling Module (Invitrogen, Carlsbad, CA). Labeled tumor and reference samples were pooled and hybridized onto an array according to the protocol. Hybridization arrays were then washed and scanned with a laser confocal scanner (Agilent Technologies). Feature Extraction software (Agilent Technologies) was used to extract the signal intensities by using the settings (44K_CGH_0605) provided by the manufacturer. CGH Analytics software (Agilent Technologies) was used for data analysis and visualization.

Gene expression levels were measured by using the Affymetrix GeneChip U133 Plus 2 system (Affymetrix, Santa Clara, CA). Sample processing and labeling were performed according to the protocol provided by Affymetrix. A total of 3 μg total RNA was used for the initial cDNA synthesis. Chips were scanned by using the GeneChip Scanner 3000 (Affymetrix).

**Statistical Analysis**

All statistical analysis was based on a two-sample test for equality of proportions with continuity correction and was performed by using a probability test on R (R Development Core Team).

**SUPPLEMENTAL DATA**

Supplemental Data include Experimental Procedures, Supplemental References, seven figures, and eight movies and can be found with this article online at http://www.devcell.com/cgi/content/full/15/3/371/DC1/.

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