Alpha 6 integrins promote cytokinesis by regulating the expression of RSK2 and MKLP1

Neetu Singh, Ph.D.¹, Hao Xu¹, Renee Thiemann¹, Kara A. DeSantis, Ph.D.^{2#},
Melinda Larsen, Ph.D.², and Susan E. LaFlamme, Ph.D.^{1*}

From the ¹Department of Regenerative and Cancer Cell Biology, Albany Medical Center, 47 New Scotland Ave, Albany NY 12208; ²Department of Biological Sciences, University at Albany, 1400 Washington Ave, Albany, NY 12222

[#]Present address: Gen*NY*Sis Center for Excellence in Cancer, Department of Environmental Health Sciences, School of Public Health at Albany, State University of New York, Albany NY

Running Title: *Integrin-RSK2 signaling regulates MKLP1 expression*

*To whom correspondence should be addressed: Susan LaFlamme: Department of Regenerative and Cancer Cell Biology, Albany Medical College Albany NY 12208; laflams@mail.amc.edu; Tel (518) 439-0840.

Keywords: Integrins, RSK2, Cytokinesis, Organ Culture

ABSTRACT

The integrin-mediated interaction of cells with components of the extracellular matrix (ECM) regulates many cellular processes including cell division. Cytokinesis is the last step of cell division and is critical for normal development and tissue homeostasis as it ensures the proper segregation of genetic and cytoplasmic material between daughter cells. Cytokinesis failure leads to defects in development and tissue differentiation, as well as tumorigenesis. Abscission of intercellular bridge that connects presumptive daughter cells is the last step of cell division. The mitotic kinesin-like protein 1 (MKLP1) plays a central role in positioning the abscission machinery. Here, we show that $\alpha 6$ integrins promote successful cytokinesis in salivary gland epithelial cells by regulating the expression of MKLP1. RNAi-mediated depletion of $\alpha 6$ integrins inhibits cytokinesis and the expression of MKLP1 and p90 ribosomal-S6-kinase 2 (RSK2). Depletion of RSK2 results in similar defects in cytokinesis and also inhibits the expression of MKLP1, suggesting that the expression of RSK2 is required downstream of integrins to promote MKLP1 expression and successful cytokinesis. RNAi-mediated depletion of RSK2 in embryonic salivary glands in organ culture also results in the inhibition of cytokinesis

and MKLP1 expression, indicating the physiological significance of this pathway.

INTRODUCTION

Integrins are α/β transmembrane receptors that mediate cell adhesion to the ECM (1). Individual cell types express different sets of integrin α/β heterodimers to bind to distinct components of the ECM. Laminins are the major ECM components expressed by epithelial cells. Alpha6 integrins, the $\alpha 6\beta 1$ and $\alpha 6\beta 4$ heterodimers, are one class of integrins expressed by epithelial cells that mediate adhesion to laminin (1). Integrin-dependent interaction of cells with components of the ECM, including laminin, activates signaling pathways to control many aspects of cell behavior, such as migration, survival, gene expression, differentiation, and proliferation, including cytokinesis (2-4).

Cytokinesis is the last step of cell division and is critical for normal development and tissue homeostasis as it ensures the proper segregation of genetic and cytoplasmic material between daughter cells (5). Cytokinesis begins with the assembly and constriction of the contractile ring that drives the ingression of the cleavage furrow, and concludes with abscission of the intercellular bridge (as referred to as the midbody) that connects presumptive daughter cells (5). Defective

or failed cytokinesis promotes tumorigenesis (6-8), whereas the deletion of genes required for cell division blocks embryonic development (9,10). Integrins impact these processes, as the loss of integrin expression or activity can lead to defective cytokinesis, resulting in defective tissue differentiation (11,12) or aneuploidy and tumor formation (13).

Much of what is known about the mechanisms that link integrins and cytokinesis have been identified using cell culture models. Our published studies identified a role for integrins in the regulation of abscission (14) and have defined a role for ERK and its downstream target the ribosomal S6 kinase (RSK) (14). RSKs are a family of serine/threonine kinases that phosphorylate many cytoplasmic and nuclear targets (15-17). We previously demonstrated that depletion of either RSK1 or RSK2 in mammary epithelial cells inhibited cytokinesis (14), but did not determine whether both RSK isoforms were activated downstream of integrins or whether RSK1 and RSK2 regulated cytokinesis by similar or distinct mechanisms. Our current study addresses these gaps in knowledge and further explores the important role of RSK2 in the successful completion of cytokinesis.

Abscission of the intercellular bridge/midbody is catalyzed by the endosomal-sorting complex required for transport (ESCRT), together with additional proteins (18). The ESCRT complex is recruited to the midbody by stepwise assembly of proteins beginning with the mitotic kinesin-like protein1, MKLP1, then the centrosomal 55 kD protein (CEP55), and Alg2-interacting protein X (ALIX) followed by the ESCRT complex (5). Here, we tested whether $\alpha 6$ integrins promote cytokinesis by regulating the expression of any of these midbody components and whether RSK1 and/or RSK2 were required for this regulation.

To test this, we first used SIMS cells (19,20), a murine salivary gland epithelial cell line that allowed us to easily examine mechanisms that regulate cytokinesis downstream of $\alpha 6$ integrins employing a cell culture model. We then confirmed the physiological significance of these mechanisms using the murine salivary gland in organ culture. We demonstrate that integrins regulate the expression of MKLP1 and that RSK2 functions downstream of integrins in the

regulation of MKLP1 expression and successful cytokinesis. We show that RSK1 and RSK2 regulate cytokinesis by distinct mechanisms and that only RSK2 functions downstream of $\alpha 6$ integrins. Lastly, we show that RSK2 also regulates cytokinesis and the expression of MKLP1 in *ex vivo* organ culture, indicating that this pathway is important not only in cell culture, but also in the more complex physiological context of the embryonic murine salivary gland.

RESULTS

Alpha 6 integrins regulate MKLP1 expression

To determine whether $\alpha 6$ integrins regulate the expression of proteins required for the assembly of the abscission machinery, we inhibited the expression of the integrin $\alpha 6$ subunit using RNAi technology targeting different regions of the $\alpha 6$ mRNA. We determined the effect of $\alpha 6$ depletion on the expression of MKLP1, CEP55, and ALIX, which are proteins involved in the recruitment of the ESCRT complex. As an initial approach, we generated a lentiviral vector for the doxycycline inducible expression of an $\alpha 6$ targeting shRNA. SIMS cells infected with the lentivirus were selected and treated with or without doxycycline. The induction of the $\alpha 6$ -targeting shRNA significantly inhibited $\alpha 6$ expression compared to control (Fig. 1A). Depletion of $\alpha 6$ integrins resulted in the inhibition of cytokinesis. We observed a significant increase in the number of cells connected by midbodies, indicative of a delay in abscission, and the accumulation of binucleated cells resulting from failed cytokinesis (Figs. 1B-C). The shRNA-dependent depletion of $\alpha 6$ integrins also results in a significant decrease in the expression of the MKLP1 protein when assayed by western blot and quantified from three independent experiments (Fig. 1D-E). Additionally, qPCR analysis indicated MKLP1 transcripts levels were also significantly decreased in $\alpha 6$ -depleted cells (Fig. 1F). In contrast, the expression of CEP55 and ALIX were not affected (Fig. 1D-F). Analogous results were obtained using an siRNA that targeted a different region of the $\alpha 6$ mRNA (Fig. 1. G-L). Thus these results indicate that $\alpha 6$ integrins regulate RNA and protein levels of MKLP1, a key cytokinesis regulator.

RSK2 functions downstream of $\alpha 6$ integrins

Although our previous studies demonstrated that both RSK1 and RSK2 regulated cytokinesis in MCF10A cells (14), these studies did not determine whether both isoforms functioned downstream of integrins or whether they regulated cytokinesis by similar mechanisms. To begin, we confirmed that both RSK1 and RSK2 regulated cytokinesis in SIMS cells. We inhibited their expression using RSK1 and RSK2 targeting siRNAs (Fig. 2A-B). As expected, depleting SIMS cells of RSK1 or RSK2 delayed abscission as indicated by an increase in cells connected by midbodies and led to the formation of binucleated cells (Fig. 2A-B).

Since both RSK1 and RSK2 inhibited cytokinesis in SIMS cells, we were interested to determine whether $\alpha 6$ integrins differentially regulated the activation or expression of RSK1 and RSK2. We first tested whether cell adhesion to laminin, a known substrate for $\alpha 6$ integrins resulted in the activation of RSK1 and/or RSK2. SIMS cells were either incubated in suspension or re-adhered to a laminin matrix for 2.5 h. RSK1 and RSK2 were then separately immunoprecipitated using isoform specific antibodies and RSK activation was evaluated by western blot using a phosphorylation-specific antibody that recognizes active RSK (15). RSK1 was not activated by adhesion; however, RSK1 was activated in response to the addition of serum (Fig. 2C). In contrast, RSK2 was activated when cells were adhered to laminin when compared with cells that were deprived of adhesion (Fig. 2D).

To determine whether $\alpha 6$ integrins differentially regulate the expression of RSK isoforms, SIMS cells were depleted of $\alpha 6$ using the inducible expression of $\alpha 6$ targeting shRNA as described above. The effect on RSK expression was analyzed by western blot and qPCR. The results indicate that depleting cells of $\alpha 6$ integrins did not affect the levels of RSK1 protein, as analyzed by western blot and quantified from three independent experiments (Fig. 2E-F). This was also true of RSK1 transcript levels (Fig. 2G). In contrast, depletion of $\alpha 6$ integrins led to the inhibition of RSK2 levels both at the protein (Fig. 2H-I) and mRNA level (Fig. 2J). Taken together our data suggest that RSK2 functions downstream of $\alpha 6$ integrins to promote successful cytokinesis.

RSK2 regulates the expression of MKLP1

As shown above, depleting cells of $\alpha 6$ integrins decreases the levels of RSK2, as well as MKLP1. This suggests that RSK2 may regulate cytokinesis by regulating the expression of MKLP1. To test this, SIMS cells were depleted of RSK2 using RNAi and the effect on the expression of MKLP1, CEP55, and ALIX was analyzed by both western blot and qPCR. Depleting cells of RSK2 inhibited the expression of MKLP1 protein, as analyzed by western blot and quantified from three independent experiments (Fig. 3A-B). As we observed with the depletion of $\alpha 6$ integrins, inhibiting the expression of RSK2 had no effect on the expression of CEP55 and ALIX (Fig. 3A-B). Additionally, qPCR analysis indicated that RSK2 depletion also inhibited the expression of MKLP1 transcripts (Fig. 3C). Similar results were obtained when RSK2 expression was inhibited using an inducible shRNA targeting RSK2 (Fig. 2D-E). In contrast, depleting cells of RSK1 did not affect the levels of MKLP1 (Fig. 1F). Taken together our results suggest that $\alpha 6$ integrins regulate cytokinesis by regulating the RSK2-dependent expression of MKLP1 and indicate that RSK1 and RSK2 regulate cytokinesis by independent mechanisms.

Depletion of MKLP1 results in similar cytokinesis defects as the depletion of $\alpha 6$ integrins or RSK2

MKLP1 is a critical regulator of cytokinesis and previous studies have demonstrated that RNAi-dependent depletion of MKLP1 results in cytokinesis failure (21). Since we propose that $\alpha 6$ and RSK2 regulate the expression of MKLP1 to promote cytokinesis, we tested whether the depletion of MKLP1 resulted in a similar level of cytokinesis failure in SIMS cells. To test this, we transfected SIMS cells with siRNA targeting MKLP1. Depletion of MKLP1 resulted in a similar level of cytokinesis failure as depletion of either $\alpha 6$ or RSK2 (Fig. 4. A-C). These results support a mechanism in which $\alpha 6$ integrins positively regulate the expression of RSK2, which in turn positively regulates the expression of MKLP1 to promote cytokinesis.

RSK2 regulates the expression of MKLP1 in the embryonic salivary gland

Much of what is known about the mechanisms that regulate cytokinesis in mammalian cells has been acquired from experiments in culture using cell lines that are easily transfected and imaged (22). However, since integrin signaling can be strongly influenced by substrate compliance (23), and our previous studies demonstrated that cytokinesis is regulated by matrix compliance in a cell type-dependent manner (24), it is important to demonstrate that pathways that regulate cytokinesis in cell culture similarly regulate cytokinesis in a more physiological complex context. Since we previously demonstrated that $\alpha 6$ integrins promote successful cytokinesis in embryonic salivary glands in *ex vivo* organ culture (14), we again used this model to test whether RSK2 regulates cytokinesis and MKLP1 expression in this context. To this end, submandibular salivary glands were isolated from mouse embryos at embryonic day 12.5 -13 (E12.5-E13) and treated for 3 days with either non-targeting or RSK2-targeting siRNA (Fig. 5A). The effects on RSK2 expression and cytokinesis were analyzed from three independent experiments. The efficiency of RSK2 knockdown was analyzed by western blot and quantified from the three experiments (Fig. 5B), which demonstrated that RSK2 depletion significantly inhibited cytokinesis. The results indicate that ~25% of cells from glands treated with RSK2 siRNA were binucleated compared with ~8% of cells from glands treated with the non-targeting siRNA (Fig. 5C). Importantly, RSK2 depletion also inhibited the expression of MKLP1 in this context, as analyzed by western blot from two independent experiments (Fig. 5D-E). Thus, RSK2 regulates cytokinesis by similar mechanisms both in salivary gland epithelial cells in culture and in embryonic salivary glands in organ culture.

DISCUSSION

In this study, we demonstrated that $\alpha 6$ integrins promote successful cytokinesis in cultured salivary gland epithelial cells, at least in part, by the $\alpha 6$ -dependent expression of MKLP1, a key regulator of cytokinesis. Our data also show that $\alpha 6$ integrins promote the expression of RSK2 and that RSK2 positively regulates the expression of MKLP1. We propose that $\alpha 6$ integrins promote a

gene expression cascade in which $\alpha 6$ integrins regulate cytokinesis by promoting the expression of RSK2, which in turn promotes the expression of MKLP1 (Fig. 6). Additionally, our results indicate that although the depletion of either RSK1 or RSK2 inhibits cytokinesis in salivary gland epithelial cells, only RSK2 is regulated by $\alpha 6$ integrins and that RSK1 and RSK2 regulate cytokinesis by distinct mechanisms.

It is well established that the compliance of the adhesion substrate impacts integrin signaling and cytokinesis (23,24). Our studies with SIMS cells were performed on culture plates, whose stiffness is orders of magnitude higher than observed in tissues (25). Therefore, it was critical to demonstrate a role for RSK2 in a more physiological context, allowing epithelial cells to interact with the ECM in a physiological compliant environment. We accomplished this by inhibiting the expression of RSK2 in embryonic salivary glands *ex vivo* organ culture. Depletion of RSK2 resulted in the inhibition of MKLP1 expression and impaired cytokinesis, confirming the significance of this pathway in a physiological context.

In addition to regulating the expression of MKLP1, our previous studies suggested that RSK activity is also required proximal to abscission, as the pharmacological inhibition of RSK after the formation of the midbody/intercellular bridge was sufficient to inhibit cytokinesis (14). We and others, have observed the localization of RSK2 in the midbody/intercellular bridge of dividing cells ((26) and N. Singh (unpublished data)), suggesting that RSK2-dependent phosphorylation of a component of the abscission machinery may promote the successful completion of cytokinesis. Thus, RSK2 may regulate cytokinesis by regulating both the expression and activity of components of the abscission machinery. RSK2 activity may also be required at late stages of cytokinesis to modulate integrin activity. Active RSK2 has been shown to down regulate integrin activity (27). Thus, the RSK2-dependent modulation of integrin activity may be required for abscission, as studies have shown that tension exerted on the intercellular bridge by presumptive daughter cells must be released for membrane fission to occur (28).

Our previous studies demonstrated that RSK2 does not regulate cytokinesis in human fibroblasts

(14), indicating the regulation of cytokinesis by RSK2 exhibits cell type specificity. Interestingly, others have identified a role for FAK-SRC signaling in promoting cytokinesis of human fibroblasts (29). The pharmacological inhibition of FAK or SRC was shown to disrupt the appropriate timing of the recruitment of CEP55 to the midbody (29), which is known to result in abscission failure (30). The ability of Src signaling to promote abscission does not appear to be cell type specific, as blocking Src signaling inhibited abscission in Hela cells (31). Hence, integrins promote cytokinesis by cell-type specific and common signaling pathways.

Multiple integrin heterodimers can promote successful cytokinesis and do so in an integrin ligand-dependent manner (14,32-35). Integrin trafficking to the cleavage furrow is important as it supports the adhesion of the cleavage furrow to the underlying matrix (34). Some mechanisms regulating integrin trafficking rely on protein interactions with the integrin $\beta 1$ cytoplasmic tail, and thus, impact cytokinesis in cells adhered to the multiple ligands for $\beta 1$ integrins (34). However, other mechanisms are specific to individual integrin heterodimers. This is the case for the $\alpha 5\beta 1$ heterodimer (35). The interaction of ZO-1 with the $\alpha 5$ subunit cytoplasmic domain is required for the trafficking of $\alpha 5\beta 1$ and cytokinesis (35). Thus, integrin-regulated cytokinesis occurs by mechanisms shared by multiple integrin heterodimers and other specific to individual integrins.

In conclusion, we have identified $\alpha 6$ integrins and RSK2 as positive regulators of cytokinesis in epithelial cells, thus, protecting epithelial tissue homeostasis, as failed cytokinesis in epithelial cells has been reported to promote tumorigenesis (6-8).

Experimental procedures

Antibodies and Reagents

Mouse monoclonal antibody to α -tubulin (DM1 α), goat polyclonal antibody to RSK2 (C-19), rabbit polyclonal antibody to CEP55 (M-300), mouse monoclonal antibody to the integrin $\alpha 6$ subunit (F6), and rabbit polyclonal antibodies to RSK1 (C-21) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit monoclonal antibody to MKLP1 (EPR10879) was from Abcam (Cambridge, UK). Mouse monoclonal antibodies

to ALIX (3A9) and GAPDH (clone GAIR) were from Thermo Fisher Scientific (Agawam, MA, USA). Rabbit polyclonal antibodies to phosphorylated serine 380 of RSK and DRAQ5 were from Cell Signaling Technology (Danvers, MA, USA). Alexa-Fluor-conjugated secondary antibodies and Hoechst 33342 were from Invitrogen. For immunofluorescence, the monoclonal antibody for α -tubulin and its corresponding secondary antibody were used at 1:800. For western blotting, antibodies were diluted to 1:500 except for antibodies to MKLP1 and pRSK, which were diluted to 1:1000.

Cell Culture

Cell lines were cultured at 37°C in 5% CO₂. The murine submandibular salivary gland epithelial cell line, SIMS cells (Laoide, 1996 and 1999), was cultured in DMEM/F12. The 804G bladder carcinoma cells (36) used to isolate laminin-332 (laminin-5) matrix and 293FT cells used in preparation of lentiviruses were cultured in DMEM. All culture media also contained 10% FBS, 100 units/ml penicillin, 100 μ g/ml streptomycin and 2.92 μ g/ml L-glutamine.

SiRNA transfection in cell culture

SIMS cells were plated in 6 well tissue culture plates and treated with siRNA (80 nM per well) using Oligofectamine (Invitrogen/ThermoFisher) following the protocol provided by the manufacturer. After 3 days, the cells were trypsinized and a fraction of the cells was used for western blot analysis. The remainder were replated on coverslips, incubated overnight and then analyzed for cytokinesis failure as described below. SiRNAs, targeting and non-targeting, were purchased from Dharmacon/Thermo Scientific. The targeting sequences are the following: RSK1 siRNA: AGGGCAAGCUCUAUCUUAU; RSK2 siRNA: GCACGAAUAGGUAGUGGAA; $\alpha 6$ siRNA: GGACCAAAGACUCGAUGUU. A SMART pool was used in the case of MKLP1 (Dharmacon, Lafayette, CO).

Generation of lentiviral vectors for shRNA expression

Sequences encoding shRNA for RSK1, RSK2 and $\alpha 6$ shRNA were cloned into the lentiviral vector, pLKO-Tet-On (Addgene plasmid # 21915) (37). The oligonucleotides encoding shRNAs were

hybridized and then cloned into the AgeI and EcoR1 sites of pLKO-Tet-On lentiviral vector. Insertion of the correct sequences was confirmed by nucleotide sequence analysis. The oligonucleotides used in vector construction are as follows: **RSK2 oligonucleotides:** 5'-CCGGGCCGTGAAGATTATTGATAAACTCG AGTTTATCAATAATCTTCACGGCTTTTGTG-3' and 5'-CGGCACTTCTAATAACTATTTGACTCAAAT AGTTATTAGAAGTGCCGAAAACTTAA-3'. **$\alpha 6$ oligonucleotides:** 5'-CCGGCGTCTGATAAAGAGAGGCTTACTCG AGTAAGC CTCTCTTTATCAGACGTTTTTG-3' and 5'-GCAGACTATTTCTCTCCGAATGAGCTCATT C GGAGAGAAATAGTCTGCAAAAACCTTCAA-3'. **RSK1 oligonucleotides:** 5'-CCGGGCTCTAT CTTATTCTGGACTTCTCGAGAAGTCCAGAA TAAGATAGAGCTTTTTTG-3' and 5'-CGAGA TAGAATAAGACCTGAAGAGCTCTTCAGGT CTTATTCTATCTCGAAAACTTAA-3'.

Lentiviruses were produced by co-transfection of 293FT cells with the expression vector together with pCMV-dR8.2 and pCMV-VSV-G and then used to infect SIMS cells. Positively infected cells were selected with 2 μ g/ml puromycin (Gibco/Life technologies). Cells were removed from puromycin 48 h prior to shRNA induction with doxycycline (100 ng/ml).

Western blotting

Western blotting was used to confirm siRNA and shRNA knockdown and to assay the effect of RSK2 depletion on various abscission components. Cells were lysed in mRIPA buffer containing phosphatase (Fisher Scientific) and protease inhibitor cocktails (Krackeler Scientific) and equal amounts of protein (30-40 μ g) were separated by SDS-PAGE and transferred to nitrocellulose and then incubated with appropriate primary and secondary antibodies. Membranes were blocked in 2% BSA/PBST for 1 h and then incubated with primary antibody application overnight at 4°C. Membranes were washed in PBST prior to secondary antibody application. Membranes were exposed to SuperSignal West Pico PLUS Chemiluminescent substrate (Thermo Scientific, #34580) and SuperSignal West Femto Maximum Sensitivity substrate (Thermo

Scientific, #34095) prior to imaging on a BioRad ChemiDoc™ MP Imaging System and analysis with Image Lab software. Protein signals were normalized to GAPDH.

Immunoprecipitation

SIMS cells were isolated from logarithmically growing cultures and either replated on a laminin-rich matrix (38,39) or incubated in suspension for 2.5 h in CCM1 medium (Thermo Scientific). After incubation, the cells were washed with 1X PBS and solubilized for 15 min in 500 μ l of lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 0.25% Deoxycholate (Na), 150 mM NaCl, 1mM EDTA, 1X protease inhibitor cocktail (Roche Applied Science), and 1X phosphatase inhibitor (PhosSTOP, Roche) on ice. Subsequent steps were performed at 4°C. Cell extracts were centrifuged for 5 min at 3000 rpm, and the supernatant was incubated for 4 h with corresponding control IgG and RSK antibody (4 μ g/ml), and then 45 μ l of protein A/G Plus Agarose beads (Pierce Biotechnology, Rockford, IL,) was added to each sample and samples were incubated for 45 min. Agarose beads/antibody complexes were recovered by centrifugation, washed five times with wash buffer (1X PBS with 1% NP40). Proteins were solubilized in SDS-PAGE sample buffer and analyzed by western blotting.

Quantitative PCR (qPCR)

RNA was isolated from SIMS cells and *ex vivo* cultures of murine submandibular salivary glands using Perfect RNA Cell Culture Kit-50 (5 Prime, Gaithersburg MD). cDNA was synthesized using iScript Reverse Transcription Supermix (BioRad) using 1 μ g of DNase-treated RNA. Equal amounts of cDNA were used in qPCR reactions performed with iQSYBER Green Supermix (BioRad). Primer sequences are provided in Table I and were identified using the Harvard PrimerBank (40-42). Ct values were normalized to β -actin. Reactions were run in duplicate in 3 independent experiments.

Immunofluorescence

Cells were cultured either on glass coverslips and fixed in 4% paraformaldehyde for 20 min or ice-cold methanol for 5 min. Cells were then permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked in 2% BSA in PBS.

Antibodies to α -tubulin to stain microtubules or DRAQ5 to stain DNA were diluted in 1X PBS. After several washes with PBS, cells were incubated with the fluorescently tagged secondary antibodies for 1 h, washed in PBS and mounted with SlowFade®Gold antifade reagent (Thermo Fisher Scientific, Agawam, MA, USA).

Microscopy

Microscopy studies were performed with a Nikon inverted TE2000-E microscope equipped with phase contrast and epifluorescence, a digital CoolSNAP HQ camera, a Prior ProScanII motorized stage and a Nikon C1 confocal system and with EZC1 and NIS-Elements acquisition software. Cytokinesis failure was quantified with 40X Nikon Cfi Plan Apo Dm oil objective.

Cytokinesis assays

To assay the inhibition of cytokinesis, we quantified the number of cells that remained connected by midbodies (intercellular bridges) or that were binucleated after cells were stained with antibodies to α -tubulin and DRAQ5. Midbodies were identified by the high concentration of α -tubulin in both arms of the midbody (intercellular bridge connecting presumptive daughter cells).

Explant cultures

Mouse submandibular salivary glands were removed and microdissected from timed-pregnant female mice (Strain CD-1, Charles River Laboratories) at embryonic day E12-E13, with the day of plug discovery designated as E0. Experiments were performed in accordance with protocols approved by the IACUC committee at University at Albany, SUNY. Explants were cultured in single-well dishes (MatTek) on top of porous polycarbonate filters (Nuclepore, Whatman WHA110405) floating on 200 μ m of serum-free DMEM/Ham's F12 medium (F12) containing ascorbic acid (150 μ g/ml), transferrin (50 μ g/ml), 100 units/ml penicillin, and 100 μ g/ml streptomycin, as reported previously (43-45).

SiRNA transfection of salivary glands in *ex vivo* organ culture

Mouse submandibular salivary glands were dissected from timed-pregnant female mice (Strain CD-1, Charles River Laboratories) at embryonic day E12-E13. The day of plug discovery designated as E0. Experiments were performed in accordance with protocols approved by the University at Albany. Three rounds of overnight transfection using 400 nM siRNA with Oligofectamine were performed for optimal knockdown. After 88 h, ten non-targeting siRNA-treated glands and ten RSK2-siRNA treated glands were lysed and analyzed for RSK2 depletion and MKLP1 expression by western blot. An additional fifteen glands from each group were disrupted by dissociating the glandular buds with dispase and collagenase followed by gravity pellet enrichment for epithelial clusters as previously described (44,46). Dissociated cells were then plated onto a laminin-rich matrix (38,39) and immunostained with antibodies to α -tubulin and Hoechst or DRAQ5 to quantify binucleated cells.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software using a two-tailed Student's t-test or one-way ANOVA with Dunnett's post-hoc analysis. P value of $p < 0.05$ was considered to be statistically significant.

Acknowledgments

The authors thank Dmitri Wiederschain (Novartis Developmental and Molecular Pathways, Cambridge, MA) for generously providing the Tet-pLKO-puro lentiviral vector, and Debbie Moran for her help with the preparation of the manuscript.

Competing interests

The authors declare no competing interests.

Funding

This work was supported by the National Institutes of Health (NIH) [Grant number GM51540 to S.E.L.]

References

1. Hynes, R. O. (2002) Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-687
2. Danen, E. H., and Sonnenberg, A. (2003) Integrins in regulation of tissue development and function. *J Pathol* **201**, 632-641
3. LaFlamme, S. E., Nieves, B., Colello, D., and Reverte, C. G. (2008) Integrins as regulators of the mitotic machinery. *Curr Opin Cell Biol* **20**, 576-582
4. Streuli, C. H. (2009) Integrins and cell-fate determination. *J Cell Sci* **122**, 171-177
5. Green, R. A., Paluch, E., and Oegema, K. (2012) Cytokinesis in animal cells. *Annu Rev Cell Dev Biol* **28**, 29-58
6. Ganem, N. J., Storchova, Z., and Pellman, D. (2007) Tetraploidy, aneuploidy and cancer. *Curr Opin Genet Dev* **17**, 157-162
7. Lacroix, B., and Maddox, A. S. (2012) Cytokinesis, ploidy and aneuploidy. *J Pathol* **226**, 338-351
8. Lens, S. M. A., and Medema, R. H. (2019) Cytokinesis defects and cancer. *Nat Rev Cancer* **19**, 32-45
9. Cook, D. R., Solski, P. A., Bultman, S. J., Kauselmann, G., Schoor, M., Kuehn, R., Friedman, L. S., Cowley, D. O., Van Dyke, T., Yeh, J. J., Johnson, L., and Der, C. J. (2011) The ect2 rho Guanine nucleotide exchange factor is essential for early mouse development and normal cell cytokinesis and migration. *Genes Cancer* **2**, 932-942
10. Wargha, R. M., Wicklund, A., Webster, S. E., and Kane, D. A. (2016) Progressive loss of RacGAP1/ogre activity has sequential effects on cytokinesis and zebrafish development. *Dev Biol* **418**, 307-322
11. Aszodi, A., Hunziker, E. B., Brakebusch, C., and Fassler, R. (2003) Beta1 integrins regulate chondrocyte rotation, G1 progression, and cytokinesis. *Genes Dev* **17**, 2465-2479
12. Lechler, T., and Fuchs, E. (2005) Asymmetric cell divisions promote stratification and differentiation of mammalian skin. *Nature* **437**, 275-280
13. Hognas, G., Tuomi, S., Veltel, S., Mattila, E., Murumagi, A., Edgren, H., Kallioniemi, O., and Ivaska, J. (2012) Cytokinesis failure due to derailed integrin traffic induces aneuploidy and oncogenic transformation in vitro and in vivo. *Oncogene* **31**, 3597-3606
14. Mathew, S. S., Nieves, B., Sequeira, S., Sambandamoorthy, S., Pumiglia, K., Larsen, M., and Laflamme, S. E. (2014) Integrins promote cytokinesis through the RSK signaling axis. *J Cell Sci* **127**, 534-545
15. Anjum, R., and Blenis, J. (2008) The RSK family of kinases: emerging roles in cellular signalling. *Nat Rev Mol Cell Biol* **9**, 747-758
16. Sulzmaier, F. J., and Ramos, J. W. (2013) RSK isoforms in cancer cell invasion and metastasis. *Cancer Res* **73**, 6099-6105
17. Cho, Y. Y. (2017) RSK2 and its binding partners in cell proliferation, transformation and cancer development. *Arch Pharm Res* **40**, 291-303
18. Elia, N., Sougrat, R., Spurlin, T. A., Hurley, J. H., and Lippincott-Schwartz, J. (2011) Dynamics of endosomal sorting complex required for transport (ESCRT) machinery during cytokinesis and its role in abscission. *Proc Natl Acad Sci U S A* **108**, 4846-4851
19. Laoide, B. M., Courty, Y., Gastinne, I., Thibaut, C., Kellermann, O., and Rougeon, F. (1996) Immortalised mouse submandibular epithelial cell lines retain polarised structural and functional properties. *J Cell Sci* **109** (Pt 12), 2789-2800
20. Laoide, B. M., Gastinne, I., and Rougeon, F. (1999) Tubular morphogenesis and mesenchymal interactions affect renin expression and secretion in SIMS mouse submandibular cells. *Exp Cell Res* **248**, 172-185
21. Matuliene, J., and Kuriyama, R. (2004) Role of the midbody matrix in cytokinesis: RNAi and genetic rescue analysis of the mammalian motor protein CHO1. *Mol Biol Cell* **15**, 3083-3094
22. Elia, N., Ott, C., and Lippincott-Schwartz, J. (2013) Incisive imaging and computation for cellular mysteries: lessons from abscission. *Cell* **155**, 1220-1231
23. Humphrey, J. D., Dufresne, E. R., and Schwartz, M. A. (2014) Mechanotransduction and extracellular matrix homeostasis. *Nat Rev Mol Cell Biol* **15**, 802-812
24. Sambandamoorthy, S., Mathew-Steiner, S., Varney, S., Zuidema, J. M., Gilbert, R. J., Van De Water, L., and LaFlamme, S. E. (2015) Matrix compliance and the regulation of cytokinesis. *Biol Open* **4**, 885-892

25. Assoian, R. K., and Klein, E. A. (2008) Growth control by intracellular tension and extracellular stiffness. *Trends Cell Biol* **18**, 347-352
26. Willard, F. S., and Crouch, M. F. (2001) MEK, ERK, and p90RSK are present on mitotic tubulin in Swiss 3T3 cells: a role for the MAP kinase pathway in regulating mitotic exit. *Cell Signal* **13**, 653-664
27. Gawecka, J. E., Young-Robbins, S. S., Sulzmaier, F. J., Caliva, M. J., Heikkila, M. M., Matter, M. L., and Ramos, J. W. (2012) RSK2 protein suppresses integrin activation and fibronectin matrix assembly and promotes cell migration. *J Biol Chem* **287**, 43424-43437
28. Lafaurie-Janvore, J., Maiuri, P., Wang, I., Pinot, M., Manneville, J. B., Betz, T., Balland, M., and Piel, M. (2013) ESCRT-III assembly and cytokinetic abscission are induced by tension release in the intercellular bridge. *Science* **339**, 1625-1629
29. Kamranvar, S. A., Gupta, D. K., Huang, Y., Gupta, R. K., and Johansson, S. (2016) Integrin signaling via FAK-Src controls cytokinetic abscission by decelerating PLK1 degradation and subsequent recruitment of CEP55 at the midbody. *Oncotarget* **7**, 30820-30830
30. Bastos, R. N., and Barr, F. A. (2010) Plk1 negatively regulates Cep55 recruitment to the midbody to ensure orderly abscission. *J Cell Biol* **191**, 751-760
31. Kasahara, K., Nakayama, Y., Nakazato, Y., Ikeda, K., Kuga, T., and Yamaguchi, N. (2007) Src signaling regulates completion of abscission in cytokinesis through ERK/MAPK activation at the midbody. *J Biol Chem* **282**, 5327-5339
32. Reverte, C. G., Benware, A., Jones, C. W., and LaFlamme, S. E. (2006) Perturbing integrin function inhibits microtubule growth from centrosomes, spindle assembly, and cytokinesis. *J Cell Biol* **174**, 491-497
33. Thullberg, M., Gad, A., Le Guyader, S., and Stromblad, S. (2007) Oncogenic H-Ras V12 promotes anchorage-independent cytokinesis in human fibroblasts. *Proc Natl Acad Sci U S A* **104**, 20338-20343
34. Pellinen, T., Tuomi, S., Arjonen, A., Wolf, M., Edgren, H., Meyer, H., Grosse, R., Kitzing, T., Rantala, J. K., Kallioniemi, O., Fassler, R., Kallio, M., and Ivaska, J. (2008) Integrin trafficking regulated by Rab21 is necessary for cytokinesis. *Dev Cell* **15**, 371-385
35. Hamalisto, S., Pouwels, J., de Franceschi, N., Saari, M., Ivarsson, Y., Zimmermann, P., Brech, A., Stenmark, H., and Ivaska, J. (2013) A ZO-1/alpha5beta1-integrin complex regulates cytokinesis downstream of PKCepsilon in NCI-H460 cells plated on fibronectin. *PLoS One* **8**, e70696
36. Izumi, K., Hirao, Y., Hopp, L., and Oyasu, R. (1981) In vitro induction of ornithine decarboxylase in urinary bladder carcinoma cells. *Cancer Res* **41**, 405-409
37. Wiederschain, D., Wee, S., Chen, L., Loo, A., Yang, G., Huang, A., Chen, Y., Caponigro, G., Yao, Y. M., Lengauer, C., Sellers, W. R., and Benson, J. D. (2009) Single-vector inducible lentiviral RNAi system for oncology target validation. *Cell Cycle* **8**, 498-504
38. Homan, S. M., Martinez, R., Benware, A., and LaFlamme, S. E. (2002) Regulation of the association of alpha 6 beta 4 with vimentin intermediate filaments in endothelial cells. *Exp Cell Res* **281**, 107-114
39. Langhofer, M., Hopkinson, S. B., and Jones, J. C. (1993) The matrix secreted by 804G cells contains laminin-related components that participate in hemidesmosome assembly in vitro. *J Cell Sci* **105 (Pt 3)**, 753-764
40. Spandidos, A., Wang, X., Wang, H., Dragnev, S., Thurber, T., and Seed, B. (2008) A comprehensive collection of experimentally validated primers for Polymerase Chain Reaction quantitation of murine transcript abundance. *BMC Genomics* **9**, 633
41. Spandidos, A., Wang, X., Wang, H., and Seed, B. (2010) PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. *Nucleic Acids Res* **38**, D792-799
42. Wang, X., and Seed, B. (2003) A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res* **31**, e154
43. DeSantis, K. A., Stabell, A. R., Spitzer, D. C., O'Keefe, K. J., Nelson, D. A., and Larsen, M. (2017) RARalpha and RARgamma reciprocally control K5(+) progenitor cell expansion in developing salivary glands. *Organogenesis* **13**, 125-140
44. Sequeira, S. J., Gervais, E. M., Ray, S., and Larsen, M. (2013) Genetic modification and recombination of salivary gland organ cultures. *J Vis Exp*, e50060

45. Daley, W. P., Gervais, E. M., Centanni, S. W., Gulfo, K. M., Nelson, D. A., and Larsen, M. (2012) ROCK1-directed basement membrane positioning coordinates epithelial tissue polarity. *Development* **139**, 411-422
46. Wei, C., Larsen, M., Hoffman, M. P., and Yamada, K. M. (2007) Self-organization and branching morphogenesis of primary salivary epithelial cells. *Tissue Eng* **13**, 721-735

Table I. qPCR Primers

Gene	PrimerBank ID	Sequence (5' 3')
Integrin α6	31982236a1	Forward: TGCAGAGGGCGAACAGAAC Reverse: GCACACGTCACCACTTTGC
β-Actin	6671509a1	Forward: GGCTGTATTCCCCTCCATCG Reverse: CCAGTTGGTAACAATGCCATGT
MKLP1	29568094a1	Forward: TTGCTGAAGTAACCCAAGAAGTG Reverse: CGGTGGGAAGCTCTGTAGAATC
CEP55	12848973a1	Forward: CCTAGTAGCTCCAAGTCAGACA Reverse: ACCTTAGGTGGTCTTTGAGTCTC
ALIX	6755002a1	Forward: TAGTGTTTGCACGGAAGACAG Reverse: GGGAGGACTGATAGGCTGGA
RSK2	29568094a1	Forward: TTAGGGCAGGGATCATTGGA Reverse: ATTTTGTCCGAACACGGTCT
RSK1	158636006c2	Forward: TCCTGGTACGCAAGGTCAC Reverse: GGAAGGCATAATGTAGCTTCACC

Fig. 1. Depletion of $\alpha 6$ integrins inhibits cytokinesis and the expression of MKLP1. (A) SIMS cells harboring an inducible shRNA targeting the integrin $\alpha 6$ subunit were treated with doxycycline to induce the expression of the $\alpha 6$ shRNA or left untreated. The efficiency of $\alpha 6$ knockdown was assayed by western blot. Shown is a representative blot probed with antibodies to the $\alpha 6$ integrin subunit and reprobed with antibodies to GAPDH as a loading control. (B) Cells expressing the shRNA or control cells were stained for α -tubulin and DNA to quantify cells connected by midbodies and (C) binucleated cells. Three hundred individual cells were analyzed from each of three independent experiments performed in triplicate. Plotted is the mean percentage of cells with midbodies or that are binucleated $\pm$ s.d. (n = 9). (D) Effect of $\alpha 6$ depletion by shRNA on the expression of midbody proteins required for cytokinesis was assayed by western blot. Shown is a representative blot. (E) Quantitation from three independent experiments $\pm$ s.d. (F) The effect of $\alpha 6$ depletion by shRNA on mRNA expression was assayed by qPCR. Data is shown from two independent experiments $\pm$ variance. (G-L) SIMS cells were transfected with either NT or $\alpha 6$ targeting siRNA. The effect of $\alpha 6$ depletion on cytokinesis (G-I) and midbody components was assayed as above for $\alpha 6$ targeting shRNA (K-L). (K) Shown is a representative blot and (L) quantitation of protein expression from three independent experiments. **p<0.05, *p<0.001.

Fig. 2. Depletion of RSK1 or RSK2 inhibits cytokinesis, but only RSK2 functions downstream of integrins. (A-B) SIMS cells were transfected with non-targeting, RSK1 targeting or RSK2 targeting siRNA. The efficiency of knockdown of RSK1 (A) and RSK2 (B) was analyzed by western blotting (left panels). The effect of depletion on cytokinesis was determined by monitoring the persistence of midbodies (middle panels) or binucleated cells (right panels). Plotted is the mean percentage of cells in $\pm$ s.d. Three hundred individual cells were analyzed from each of three independent experiments performed in triplicate (n = 9). (C-D) Adhesion-dependent activation of RSK1 and RSK2 was determined by incubating cells either in suspension (Sus) or re-adhered (Ad) to a laminin matrix. (C) RSK1 and (D) RSK2 were immunoprecipitated with isoform specific antibodies and analyzed for RSK activation by western blot using an activation-specific antibody (pRSK) and then reprobed for RSK1 (C) or RSK2 (D). Although RSK1 was not activated upon adhesion it was activated by the addition of serum (S) compared to serum free medium (SF) (C). (E-F) The effect of depletion of $\alpha 6$ on the expression of RSK1 (E) and RSK2 (H) was analyzed by western blotting. Protein expression was quantified from three independent experiments (F&I). The effect of $\alpha 6$ depletion on RSK1 (G) and RSK2 (J) RNA levels was analyzed by qPCR. Plotted is mean $\pm$ variance from two independent experiments. *p<0.05, **p<0.001.

Fig. 3. RSK2 inhibits the expression of MKLP1 protein and mRNA transcripts. SIMS cells were transfected with RSK2 or RSK1 siRNAs. (A-B) The effects of RSK2 depletion on α6 and MKLP1 and other cytokinesis proteins were assayed by western blot. (A) Shown is a representative blot and (B) quantitation from three independent experiments ± s.d. (C) The effect of RSK2 depletion on mRNA expression was assayed by qPCR. Plotted is the mean ± variance from two independent experiments. (D-E) Inhibiting the expression of RSK2 by a doxycycline inducible shRNA results in similar effects and inhibited the expression of MKLP1 protein and transcripts (F) The siRNA-dependent depletion of RSK1 did not affect the expression of MKLP1. **p < 0.001

Fig. 4. Depletion of MKLP1 inhibits cytokinesis in SIMS cells. (A-C) SIMS cells were transfected with MKLP1 siRNA. (A) The efficiency of depletion was determined by western blot. The effect of MKLP1 depletion of cytokinesis on the persistence of midbodies and the formation of binucleated cells was analyzed. Plotted is the mean percentage of cells with midbodies (B) or that are binucleated (C) $\pm$ s.d. Three hundred cells were analyzed from each of three independent experiments performed in triplicate. ** $p < 0.001$, *** $p < 0.0001$.

Fig. 5. Depletion of RSK2 inhibits cytokinesis and MKLP1 expression during salivary gland morphogenesis in *ex vivo* organ culture. (A) Shown is a schematic of the experimental design. (B-C) Submandibular salivary glands were treated with RSK2-targeting or non-targeting siRNA starting at E12.5. (B) The efficiency of RSK2 knockdown was assayed by western blot and (C) quantified from three independent experiments to determine the effects on cytokinesis. Cytokinesis was assayed in 15 glands from each treatment, and approximately, 100 cells were analyzed per sample from each of three independent experiments. Plotted is the mean percentage of binucleated cell $\pm$ s.d. (D) Representative western blots of MKLP1 expression in glands treated with NT- or RSK2-targeting siRNA. (E) Quantitation of data from 2-independent experiments plotted as the mean $\pm$ variance. **p<0.001, ***p<0.0001.

Fig. 6. Model for the regulation of cytokinesis by $\alpha 6$ integrins.