Depletion of nuclear actin is a key mediator of quiescence in epithelial cells

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Summary

Functional differentiation is orchestrated by precise growth-regulatory controls conveyed by the tissue microenvironment. Cues from laminin 111 (LN1) lower transcription and suppress mammary epithelial cell growth in culture, but how LN1 induces quiescence is unknown. Recent literature points to involvement of nuclear β-actin in transcriptional regulation. Here, we show that quiescence induced by growth factor withdrawal, or LN1 addition, rapidly decreases nuclear β-actin. LN1, but not other extracellular matrix (ECM) molecules, decreases the levels of nuclear β-actin and destabilizes RNA polymerase (RNA Pol) II and III binding to transcription sites, leading to a dramatic drop in transcription and DNA synthesis. Constitutive overexpression of globular β-actin in the nucleus reverses the effect of LN1 on transcription and RNA Pol II association and prevents the cells from becoming quiescent in the presence of LN1. The physiological relevance of our findings was verified by identifying a clear spatial separation of LN1 and β-actin in developing mammary end buds. These data indicate a novel role for nuclear β-actin in growth arrest of epithelial cells and underscore the importance of the integrity of the basement membrane in homeostasis.

Key words: Actin, Laminin, Microenvironment, Nucleus

Introduction

The establishment and maintenance of the differentiated state is tightly coordinated with growth arrest, a process controlled by cues from the tissue microenvironment (Bissell et al., 2005; Lian et al., 2004; Spencer et al., 2007). We and others have used mammary epithelial cells in vivo and in culture to explore the mechanisms involved in tissue-specific gene expression because this cell type fluctuates between periods of quiescence and active growth in response to changes in hormones and other microenvironmental effectors throughout the reproductive life of a female (Ferguson et al., 1992; Keely et al., 1995; Stoeckelhuber et al., 2002; Woodward et al., 2001). During puberty, the ducal epithelium extends through the mammary fat pad into an arborous network. The extending tips of this network, or terminal end buds, comprise highly proliferative epithelial cells supported by branching ducts of relatively quiescent cells (Russo and Russo, 1978; Williams and Daniel, 1983). A basement membrane (BM) rich in laminin 111 (LN1) and collagen IV (Col IV) envelops the ducts but is essentially absent near the proliferative front (Klinowska et al., 1999; Russo and Russo, 1978; Williams and Daniel, 1983).

Using three-dimensional (3-D) cell culture models, we had shown that, in LN1-rich matrices in culture, nonmalignant mammary cells from both mouse and human form polarized, growth-arrested acini (Barcellos-Hoff et al., 1989; Petersen et al., 1992) and that LN1 induces changes in transcription factor translocation, chromatin organization and mRNA expression, events important for growth arrest and mammary-specific functions (Le Beyec et al., 2007; Myers et al., 1998; Plachot and Lelievre, 2004; Xu et al., 2009; Xu et al., 2007). LN1 is known to induce changes in the organization of cytoskeletal actin (Roskelley et al., 1994), and previous reports have described an essential role for cytoplasmic actin in the functional differentiation and branching morphogenesis of mammary epithelial cells (Akhtar and Streuli, 2006; Alcaraz et al., 2008; Ewald et al., 2008; Moore et al., 2005; Roskelley et al., 1994). By comparison with cytosolic actin, the possible contributions made by nuclear actin in functional differentiation and ECM signaling are not well established. Nuclear actin was shown recently to be important for transcriptional activity and chromatin organization in several cell types from other organisms (Chuang et al., 2006; Dundr et al., 2007; Jockusch et al., 2008; Schleicher and Jockusch, 2008). In addition, studies on Xenopus oocytes showed that nuclear actin plays an important role in chromosome congression and nuclear envelope assembly (Krauss et al., 2003; Lenart et al., 2005) – events essential for cell division. We hypothesized that there might be a connection between nuclear β-actin and growth control and that β-actin might be an important mediator of LN1 signals to control epithelial cell quiescence.

Here, we report that induction of epithelial cell quiescence by addition of LN1 or removal of growth factors leads to rapid downmodulation of nuclear β-actin, destabilization of RNA Pol II and III binding to transcription sites and cessation of DNA synthesis. Overexpression of β-actin in the nucleus opposes growth arrest by LN1. In the developing mammary end bud, high levels of β-actin and transcription are localized essentially to the regions of growth, where there is little or no LN1 deposition. Our results identify LN1 as a physiological regulator of nuclear and cytoplasmic β-actin levels in mammary epithelial cells and implicate loss of nuclear β-actin as a key causal step for quiescence in mammary epithelial cells.
Results

Growth and quiescence correlate with nuclear β-actin levels

To investigate the relationship between the levels of nuclear β-actin and growth control, we examined an asynchronous population of proliferating cells and observed a dramatically higher level of nuclear β-actin in cells that were actively synthesizing DNA compared with those that were not (Fig. 1A). Furthermore, cells growth arrested by depletion of growth factors displayed universally lower nuclear β-actin than those that were actively proliferating (Fig. 1B), suggesting a correlation between quiescence and reduction in nuclear β-actin levels.

To determine whether growth arrest induced by LN1 treatment similarly involved the depletion of nuclear β-actin, we measured the effect of this ECM molecule on DNA synthesis as a function of time. Treatment with soluble LN1 led to a decrease in both the levels of endogenous nuclear β-actin and DNA synthesis after only 2 hours (Fig. 1C,D). A discernible effect on total cellular β-actin levels occurred after 4 hours (supplementary material Fig. S1). By 8 hours, DNA synthesis, as well as the levels of nuclear and cytoplasmic β-actin, showed a decrease (Fig. 1E,F). Note that LN1 treatment reduces nuclear and cytoplasmic YFP–β-actin, but not YFP alone, compared with control cells cultured in the absence of ECM for 24 hours and that nuclear β-actin levels start to decline significantly before cytoplasmic β-actin levels. NSD, no significant difference. Scale bars: 10 μm.
cytoplasmic β-actin, had begun to stabilize to about 15% of those observed in untreated cells (Fig. 1C,D and supplementary material Fig. S1) and remained low for an additional 48 hours (data not shown). Culturing mammary epithelial cells on top of an insoluble LrECM gel for 24 hours also resulted in a downmodulation of both cytoplasmic and nuclear actin (supplementary material Fig. S2). These observations suggest that the effect of LN1 on nuclear actin occurs independent of its state of solubility.

The differential and temporal effect of LN1 on the cytoplasmic and nuclear β-actin population was confirmed by measuring the effect of this ECM molecule on an exogenously expressed, CMV-promoter-driven, transgenic reporter comprising YFP tagged to the N-terminal end of the β-actin open reading frame (Fig. 1E). To ensure that the measured effect of LN1 was specific for β-actin rather than the transgene reporter, we measured YFP levels in both the cytoplasm and the nucleus of cells transiently transfected with an identical CMV-promoter-driven vector deleted for the β-actin open reading frame. Under the latter conditions, the YFP levels did not change in response to LrECM treatment (Fig. 1F). Thus, LN1-induced reduction of the nuclear and cytoplasmic β-actin pools occurs through posttranscriptional events.

**The levels of β-actin are high in terminal end buds and correlate inversely with LN1 localization**

That the outermost edge of the terminal end bud is a highly proliferative region of the epithelial network in the developing mammary gland is well established (Russo and Russo, 1978; Williams and Daniel, 1983). To confirm the physiological significance of our culture findings, we examined the localization and levels of β-actin and LN1 in terminal end buds of the mammary gland from virgin 6–10-week-old BALB/c mice. We observed that the tip of the terminal end bud was greatly enriched in cells containing high levels of β-actin (Fig. 2A). Terminal end buds labeled with fluorophore-tagged DNase 1, a nuclease shown to both bind to globular actin in a 1:1 ratio (Haugland et al., 1992), showed an enrichment pattern similar to that of actin (Fig. 2B). By contrast, epithelial cells in LN1-rich regions at the relatively quiescent trunk of this developmental structure stained weakly for β-actin (Fig. 2B).

LrECM and the BM surrounding epithelial cells in the terminal end bud contain LN1 and Col IV. To confirm that LN1 is the main ECM component affecting β-actin levels in the mammary gland, we treated YFP–β-actin-transfected mammary cells for 48 hours with molar amounts of purified LN1 or Col IV equivalent to those in LrECM (Novaro et al., 2004). In addition, we treated cells with fibronectin because expression of this ECM molecule is upregulated during development of the mammary gland (Woodward et al., 2001). Only treatment with LN1 elicited a response that was similar to that observed after LrECM treatment (Fig. 2C, Fig. 1E).

The generality of the effect of LN1 treatment on nuclear β-actin was tested on two additional mouse mammary epithelial cell lines and a nonmalignant, spontaneously immortalized human breast epithelial cell line. In all three cases, LN1 treatment substantially decreased the levels of endogenous β-actin without affecting the levels of lamin A and C (supplementary material Fig. S3, and data not shown). These results indicate that LN1 is the main LrECM component affecting the levels of β-actin and growth of mammary epithelial cells and that these effects are not cell line- or species-specific.

**Increasing globular β-actin in the nucleus by constitutive expression of a NLS–β-actin or NLS–β-actin R62D transgene opposes LN1-mediated growth arrest**

To establish a mechanistic link between nuclear β-actin levels and growth arrest, we stably transfected cells with a constitutively expressed, FLAG-tagged NLS–β-actin transgene (Vartiainen et al., 2007) and generated low- and high-expressing clonal cell populations. The clonal cells expressing high transgene levels incorporated twice as much bromodeoxyuridine (BrdU) after

![Fig. 2](image-url)
2 hours of LN1 treatment compared with the low-expressing cells (Fig. 3), confirming that the level of nuclear β-actin is functionally important for growth control.

As much as 80% of the nuclear actin pool exists in a globular state (McDonald et al., 2006), and previous studies have shown that globular β-actin plays an important role in the activities of RNA Pol I, II and III (Hofmann et al., 2004; Hu et al., 2004; Kukalev et al., 2005; Philimonenko et al., 2004). Our analysis of mammary gland tissue sections showed a strong positive correlation between DNase-labeled globular actin and regions of epithelial cell growth (Fig. 2B). We generated clonal populations of cells constitutively expressing low and high levels of FLAG-tagged NLS-β-actin R62D, a mutant form that remains globular and is unable to polymerize (Posern et al., 2002). Clones with low expression of the NLS-β-actin R62D transgene displayed a capacity for BrdU incorporation in the presence of LN1 that was essentially similar to that displayed by clones with low NLS-wild-type (WT) β-actin levels (Fig. 3). Similarly, clones with high NLS-β-actin R62D or NLS-WT β-actin levels displayed a comparable capacity for BrdU incorporation in the presence of LN1 (Fig. 3B). Thus, the growth-promoting effect of nuclear β-actin is mediated predominantly by its globular form.

**Nuclear β-actin colocalizes significantly with transcription foci**

To determine whether nuclear β-actin plays an active role in transcriptional events important for growth in the terminal end bud, we performed an in vivo nuclear run-on transcription assay for which, before sacrifice, we injected the mammary gland of 6–10-week-old BALB/c mice with 5-fluorouridine (FU), a label for de novo RNA synthesis, and visualized FU-labeled RNA by immunofluorescence (IF). This analysis showed that the tip of the terminal end bud is enriched in cells with high levels of FU incorporation (Fig. 4A).

An in-culture nuclear run-on assay showed that ~50% of endogenous nuclear actin was organized nonrandomly and significantly colocalized with sites of RNA Pol activity in the

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**Fig. 4.** There is a statistically significant colocalization of nuclear β-actin with transcription foci. (A) Immunofluorescence localization of FU incorporation sites in a tissue section of a terminal end bud from pre-pubertal mouse mammary gland. Note that the localization of F-RNA is similar to globular β-actin, but both are inversely correlated with the LN1 localization shown in Fig. 2B. (B) (i) Nuclei from ScP2 cells cultured under growth conditions were analyzed by immunofluorescence for endogenous (endog.) β-actin colocalization with F-RNA (left). (ii) Graph displays each nucleus as a circle and the percentage of β-actin nonrandomly colocalized with F-RNA per nucleus (yellow in image; n=39). All colocalization values were calculated as described in the Materials and Methods section and are statistically significant (P<0.05). (C, D) Quantification of F-RNA incorporation in ScP2 cells in response to LrECM treatment over time showing that LnECM treatment causes a dramatic decline in transcriptional activity over 24 hours compared with that of control cells. Graphs represent: (C) the mean ± s.d. pixel intensity and (D) nuclear volume of F-RNA transcription foci over LrECM treatment time (n=64–114 nuclei per time-point). Values are representative of two independent experiments. Abbreviation: a.u., arbitrary units. Student’s t-test (95% confidence interval). (E) F-RNA incorporation in cells treated for 48 hours with LnECM, collagen IV (Col IV) or laminin 111 (LN1) showing that it is the LN1 component of LrECM that represses transcriptional activity. Images are overlaid with white trace contours of corresponding DAPI images to delineate the limits of each nucleus. Scale bars: 50 μm (A); 2 μm (B); 10 μm (E).
nucleoplasm of proliferating cells (Fig. 4B). We confirmed also by co-immunoprecipitation (co-IP) that β-actin and RNA Pol II interact with one another in mammary epithelial cells (supplementary material Fig. S4). Additional nuclear run-on assays showed that LrECM treatment caused a progressive decrease in the intensity and volume of labeled transcription foci over time (Fig. 4C,D). Again, we confirmed that LN1 was the main component in LrECM mediating this effect by treating cells for 48 hours with LrECM or with molar equivalents of purified LN1 or Col IV. LN1 reduced FU incorporation dramatically, whereas Col IV did not (Fig. 4E).

**Increased nuclear globular β-actin level by constitutive expression of a NLS-β-actin R62D transgene opposes LN1-mediated transcriptional repression**

A strong link between the effects of LN1 on nuclear β-actin and RNA Pol activity was established by testing the outcome of constitutive expression of NLS-β-actin R62D on LN1-mediated transcriptional repression. The association of this mutant β-actin isoform with transcription sites was confirmed by colocalization analysis. Approximately 40% of the NLS-β-actin R62D protein was nonrandomly colocalized with RNA Pol II in proliferating cells, and this association was statistically significant (Fig. 5A). Quantification of the nuclear volume of FU label in NLS-β-actin R62D-transfected cells after treatment with LN1 showed that expression of this construct increased the volume of incorporated label compared with that in cells transfected with the NLS vector control (Fig. 5B). Interestingly, cells that were made to overexpress high levels of exogenous nuclear actin did not display a significant increase in transcriptional or growth activity in comparison with low-expressing cells when cultured under routine growth conditions (supplementary material Fig. S5).

**LN1 treatment destabilizes RNA Pol II and III interactions with the nuclear substructure**

The majority of general transcription factors, active RNA Pol II molecules and sites of transcription are physically associated with what is referred to as the nuclear substructure, nuclear matrix or nuclear scaffold and remain so even after detergent-extraction and chromatin removal (Kimura et al., 1999). We measured the kinetics of LrECM-dependent changes in RNA Pol levels in whole-cell lysates and in the Triton X-100 (Triton)-resistant fraction of the nucleus that contains the nuclear substructure. Whole-cell RNA Pol levels remained constant after 4 hours of LrECM treatment (data not shown), but the fraction of RNA Pol III and II molecules associated with the nuclear substructure after LrECM treatment decreased by ∼50% by 2 hours (Pol III) and 4 hours (Pol II) (Fig. 6A,B). By contrast, Triton-resistant RNA Pol I levels did not change significantly even after 4 hours (data not shown).

Actin has been shown to associate directly with all three RNA polymerases (Hofmann et al., 2004; Hu et al., 2004; Philimonenko et al., 2004) and is required for the interaction of RNA Pol II and chromatin remodeling factors with the nuclear substructure (Andrin and Hendzel, 2004; Hofmann et al., 2004; Zhao et al., 1998). Our results (Fig. 5B) showed that constitutive expression of NLS-β-actin R62D confers resistance to LN1-mediated transcriptional repression. We asked whether NLS-β-actin R62D would confer resistance to LN1-mediated RNA Pol destabilization. We observed that the volume occupied by RNA Pol II or RNA Pol II P-Ser5, the form of RNA Pol II engaged in transcriptional initiation (Corden, 2007; Komamitsky et al., 2000), was significantly larger in cells expressing NLS-β-actin R62D compared with cells transfected with the NLS vector control (Fig. 6C, and data not shown). These findings, combined with the results of the RNA Pol II solubility experiments (Fig. 6B), indicate that the attenuation of transcription by LN1 is mediated through a decrease in nuclear β-actin levels.

**Nuclear β-actin levels are downmodulated through inhibition of PI3K, but not MEK1 or SRC**

Additional insight into the signaling network that might control nuclear β-actin levels was gained by treating cells with inhibitors of signaling molecules such as Src (PP2), phosphoinositide 3-kinase (PI3K: LY294002) and MEK1 (PD98059), a number of which were shown previously to be operative in morphogenesis of the mammary gland (Fata et al., 2007; Liu et al., 2004). Only treatment with the inhibitor for PI3K significantly reduced the levels of nuclear β-actin (supplementary material Fig. S6), indicating a connection between this pathway and actin levels in the nucleus.

**Discussion**

The presence of actin in the nucleus, long a controversial subject, is now well established (Gieni and Hendzel, 2009; Hofmann and de Lanerolle, 2006; Pederson and Aebl, 2005; Schleicher and Jockusch, 2008). Similarly, the important signaling role of the ECM in regulation of tissue-specific functions has been demonstrated clearly (Bissell et al., 2005; Brill et al., 2002; Damsky and Illic, 2002; Franceschi and Xiao, 2003; Nelson and Bissell, 2006; Spencer et al., 2007). It is also appreciated that cytoplasmic actin is involved in transmission of ECM signals to the nucleus (Akhtar and Streuli, 2006; Alcaraz et al., 2008). Here, we demonstrated that nuclear β-actin is an important player in the induction of epithelial cell quiescence initiated by the presence of...
LN1 or growth factor withdrawal. Focusing on LN1 as the most likely component of the basement membrane that signals for quiescence in vivo, we show that, in mammary epithelial cells, nuclear actin pools, proliferation and transcriptional activity are all highly correlated, and coordinately downmodulated, in an early response to LN1. Forcing globular nuclear actin levels to remain high opposes LN1 attenuation of growth and maintains cells in a proliferative state. These findings, combined with our observation that cells with high actin and transcription levels are spatially excluded from LN1-rich regions in the mammary glands of virgin mice, suggest strongly that reduction of nuclear actin levels is a requirement for epithelial cell quiescence.

It is important to note that overexpression of exogenous nuclear actin did not cause a significant increase in transcriptional or growth activity in cells cultured under routine growth conditions. We attribute this to the fact that only a fraction of nuclear actin colocalized with transcription and RNA Pol II sites in actively proliferating cells, and therefore a sufficient amount of endogenous nuclear actin was already available to these sites. However, when wild-type cells were treated with LN1, the amount of endogenous nuclear actin ultimately fell by ~90%, to a level that is well below the ~30% that is localized with transcription sites (Fig. 4). Such an effect almost certainly influenced nuclear actin protein levels at transcription sites and probably explains why artificially elevating nuclear actin levels through expression of the NLS–actin transgene did not cause a significant increase in transcriptional or growth activity. In cells cultured under routine growth conditions, nuclear actin pools, proliferation and transcriptional activity are all highly correlated, and coordinately downmodulated, in an early response to LN1. Forcing globular nuclear actin levels to remain high opposes LN1 attenuation of growth and maintains cells in a proliferative state. These findings, combined with our observation that cells with high actin and transcription levels are spatially excluded from LN1-rich regions in the mammary glands of virgin mice, suggest strongly that reduction of nuclear actin levels is a requirement for epithelial cell quiescence.

A number of earlier studies had presented data compatible with the idea that epithelial cell growth and laminin expression might be inversely correlated (Barcellos-Hoff et al., 1989; Bernfield and Banerjee, 1972; Mollard and Dziadek, 1998; Moore et al., 2002; Petersen et al., 1992). We and others have demonstrated that addition of LrECM, or LN1 alone, leads to growth arrest of mammary epithelial cells and induces dramatic changes in cell shape and cytoskeletal actin organization, events required for mammary-specific gene expression (Akhtar and Streuli, 2006; Alcaraz et al., 2008; Le Beyec et al., 2007; Lelievre et al., 1998; Roskelley et al., 1994; Weaver et al., 1997; Zoubiane et al., 2004). But none of these studies had explored the connection of LN1 to growth-regulatory events in the nucleus or to nuclear actin.

Evidence compiled from Streuli and colleagues (Akhtar et al., 2009; Akhtar and Streuli, 2006), as well as our laboratory (Xu et al., 2010), suggests that the Rac1–PI3K signaling pathway provides an important link between LN1–integrin adhesion and mammary-specific function. Our data suggest a scenario in which LN1 signals through PI3K to initiate a cascade of events that culminate in quiescence: the first events measured here are a reduction in the levels of nuclear β-actin, RNA-Pol-III-mediated transcription and DNA synthesis, followed by a decrease in RNA Pol II activity and alterations in the cytoplasmic β-actin network (Fig. 7A,B). In detail, we show that LN1 inhibits transcription by destabilizing the association of RNA Pol II and III (but not Pol I) with sites of transcription. Importantly the effect of LN1 on RNA Pol II and cell growth can be overcome through overexpression of globular β-actin.

Prior work has demonstrated a clear dependence on RNA Pol III and II for proliferation. Disruption of RNA Pol III function in zebrafish decreases tRNA levels and induces cell cycle arrest in hyperproliferative larval tissues (Yee et al., 2007), and treatment with α-amanitin, a potent and highly specific inhibitor of this polymerase, prevents fibroblasts from entering S phase (Adolph et al., 1993). Although a direct connection between the effects of LN1 on nuclear β-actin levels and RNA Pol III remains to be elucidated further, a causal connection is highly likely considering that nuclear β-actin is essential for basal transcription by RNA Pol III (Hu et al., 2004).
An evolutionary conservation of a nuclear actin-dependent growth-control mechanism for epithelial cells is suggested by our parallel results in murine and human cells. Indeed, actin has been identified in the nucleus of cells isolated from many different organisms spanning the evolutionary tree (Jockusch et al., 2006). Observations that actin exists also in the nuclei of other epithelial cell types (Jockusch et al., 2006) raise the possibility that nuclear actin might mediate cues from specific ECM and growth factor molecules that would regulate the development and functions of other organs.

The extracellular cues that govern growth and differentiation undoubtedly are context dependent and cell-type specific. Indeed, prior work has shown nuclear actin to play an inhibitory role in the transcriptional activation of genes targeted by serum-response factors in NIH 3T3 fibroblasts (Vartiainen et al., 2007). As different organs have tissue-specific patterns of ECM deposition, the role of nuclear actin in gene regulation and homeostasis is most likely a general phenomenon modulated by cell and tissue context.

It is interesting to note that mitotic spindle orientation and cell polarity, morphogenetic phenotypes that are fundamental for development, are guided also by the extracellular matrix, and mediated through the actin cytoskeleton (Thery et al., 2005; Toyoshima and Nishida, 2007). Nuclear actin has been shown to exist in both filamentous and globular states in human epithelial cells (McDonald et al., 2006), and the demonstration that the mechanical tug of an integrin receptor can induce changes in cytoskeletal and nuclear organization along the axis of applied tension (Maniotis et al., 1997) suggests that both nuclear and cytoplasmic actin unify and integrate the response of a cell to signaling from the ECM. Prior studies have shown that treatment with LN1 decreases the levels of actin stress fibers (Roskelley et al., 1994), promotes formation of a cortical actin network (Roskelley et al., 1994) and decreases cellular elasticity through downregulation of actin polymerization and myosin II activity (Alcaraz et al., 2008). These tensile and structural changes are required for mammary gland function (Akhtar et al., 2009; Akhtar and Streuli, 2006; Alcaraz et al., 2008; Roskelley et al., 1994). Thus, it is tempting to speculate that LN1-mediated changes in the nuclear and cytoplasmic actin networks alter the signaling networks in epithelial cells to favor events important for differentiation. Such a continuum would fulfill an important tenet of the model of ‘dynamic reciprocity’ (Bissell et al., 1982) and help to describe how specific ECM molecules might control gene expression in different tissues.

It remains to be determined whether dysregulation of the levels or organization of nuclear actin is responsible for the inability of malignant cells to respond to growth-inhibitory signals from LN1 (Petersen et al., 1992). Our preliminary results point to this direction. In addition, the finding that LN1 expression is lost in the myoepithelial cells isolated from human tumors (Gudjonsson et al., 2002) places the interaction of LN1 and breast tumor cells at the forefront of future investigations.

Fig. 7. Schematic model of the sequence of LN1-initiated events that give rise to quiescence. (A) Exposure of mammary epithelial cells to LN1 induces a dramatic decline in the levels of nuclear β-actin, nuclear matrix (NM)-associated RNA Pol III and DNA synthesis by 2 hours. By 4 hours, the levels of both NM-bound RNA Pol II and cytoplasmic β-actin also begin to decline. It is important to note that these pools are not completely depleted. The populations of nuclear β-actin, Pol II and Pol III remaining after growth arrest are probably required for LN1-induced tissue-specific gene expression. CNTRL, control. (B) Working model showing that LN1 engagement with cell surface receptors such as dystroglycan (DG) or integrin (Int) influences phosphoinositide 3-kinase (PI3K) activity in a manner that decreases nuclear β-actin levels, DNA and RNA synthesis and RNA Pol III binding to the nuclear matrix. These events would work in concert to decrease the level of nuclear-matrix-associated RNA Pol II, resulting in an additional decrease in RNA synthesis that is either contributory to, coincidental with or a consequence of a reduction in cytosolic β-actin levels. The nature of the detailed interactions between these events remains to be elucidated. FAK, focal adhesion kinase.

Materials and Methods
Expression plasmids and antibodies
The constructs for eYFP and eYFP-human-β-actin were from Clonetech. The FLAG-tagged NLS-β-actin constructs were kind gifts from Richard Treisman (London Research Institute, UK). The NLS-wild-type-β-Actin construct was used to generate the FLAG-tagged NLS vector control. YFP–LAP was generated from CMV-LAP, which was kindly provided by Ueli Schibler (University of Geneva, Switzerland). The following antibodies were used: rabbit-anti-lamin A/C (Santa Cruz 1/500), -anti-β-actin (ab1801 from Abcam; used for immunolabeling experiments at 1/100), -anti-fibrinogen (Novocastra) and –anti-fetal calf serum (Chemicon International).
plastic dishes and treated for 2, 4, 8, 24 or 48 hours with DM supplemented with or without ECM (10 μM). Cells were fixed with paraformaldehyde (PFA: Electron Microscopy Sciences) for 5 minutes, permeabilized with PBS–0.5% Triton X-100 (v/v) for 10 minutes, stained with DNase I-594 for 45 minutes and then counterstained with DAPI. For staining intensity levels of NLS–β-actin (Costes et al., 2004) were measured using Imaris and used to assess the nonrandom distribution of NLS–β-actin within nuclei. Immunolocalization was performed similarly on cryopreserved sections of thickness 5μm that were isolated from tissue collected from 6-week-old mice. Cryopreserved tissue also was stained with antibodies against actin, Pol II or BrdU. Sites of BrdU labeling were immunolabeled in cells cultured under normal growth conditions or in the presence of LN1 for 2 hours using a kit supplied by the label manufacturer (Roche).

Primary antibodies were immunolabeled with fluorochrome-conjugated goat secondary antibodies in blocking buffer, and nuclei were stained with DAPI. For NLS–β-actin and RNA Pol II colocalization studies, transfected cells were co-immunolabeled with primary antibodies to RNA Pol II and FLAG. For biochemical studies analyzing the effect of ECM molecules on endogenous β-actin levels, cells were seeded at a density of 5×10^5/cm^2 or 7.5×10^4/cm^2 onto 10 cm^2 dishes and treated for 2, 4, 8, 24 or 48 hours with DM supplemented with or without 2% (v/v) α-amanitin. NLS–β-actin levels, nuclear isolation procedures. Wild-type- or NLS- or NLS–β-actin-transfected cells were fixed with 1% PFA and then DNase I (Sigma) to 0.25 kU/ml and the sample was incubated on ice for 10 minutes. Insoluble particulates were removed from the cell suspension by centrifugation at 10,000 g for 10 minutes. Protein complexes containing FLAG-tagged NLS–β-actin were isolated by incubating the supernatant with M2 antibody against FLAG conjugated to agarose beads (Sigma). Proteins nonspecifically bound to the sepharose were washed away with rinsing buffer (Xu et al., 2007), and the remaining antibody–protein complexes were eluted from the sepharose with SDS loading buffer.

Nuclear run-on assays

Labeling of de novo synthesized RNA in cells in culture was performed as described previously (Bloseo et al., 2001; Bossert et al., 2000; Faro-Trindade and Cooke, 2006). In brief, cells were incubated without ECM, as described above, and then treated at 37°C with 6 mM FU (Sigma) for up to 30 minutes or 500 mM bromouridine triphosphate (Sigma) for 7 minutes. Label incorporation was terminated by fixation with 1% PFA for 5 minutes, which was subsequently quenched with PBS–25μM glycine. For the labeling of nuclei, cells were permeabilized in 0.5% or 1% Triton–PBS for 10 minutes, blocked with 100 μg/ml LN1 (Sigma), 20 μg/ml collagen IV (BD) or 20 μg/ml fibronectin (BD). We used LeFcm for all our studies, and DNA from transfected cells performed to investigate the kinetics of LN1 on β-actin or transcription as a less-expensive substitute for purified LN1. wild-type, YFP–β-actin- or NLS–β-actin-transfected cells were fixed with PFA or methanol/aceton (1:1; v/v). PFA reactivity was terminated with PBS–25 mM glycine and cells were permeabilized with PBS–0.5% Triton X-100 (v/v) for 5 minutes (YFP), or 1% Triton X-100 (v/v) for 10 minutes (DAPI) and mounted for imaging. For immunolabeling of nuclear actin, Pol II or transcription sites, wild-type cells treated with or without ECM were incubated in blocking buffer (500 mM bromouridine triphosphate in PBS or 5% goat serum in PBS) for 20 minutes at RT, and then incubated in blocking buffer supplemented with antibody against BrdU. Cells were rinsed with PBS containing 0.1% Tween-20, incubated in blocking buffer supplemented with secondary antibody, washed with PBS, stained with DAPI (Sigma) and then mounted with Vectashield (Vector) for confocal microscopy.

For detecting sites of de novo RNA synthesis in the mouse mammary gland, a 0.4 M solution of FU in 0.9% saline was injected peritoneally at a dose of 5 μl/g into 10-week-old BALB/c mice, as described previously (Chadwick and Rogers, 1972), and allowed to incorporate into transcription sites for 30 minutes. Mice were then sacrificed, and the glands were excised and embedded in optimal cutting temperature (OCT) medium (Tissue-Tek). The cryo-preserved tissue was sectioned into sections of thickness 30 μm, fixed with 2% PFA, incubated in PBS–125 mM glycine, permeabilized with 0.5% Triton, blocked with 5% goat serum in PBS for 1 hour and immunolabeled with an antibody against BrdU as described above. LN1 α-actin chain immunolocalization was performed similarly on cryopreserved sections of thickness 30 μm collected from 6-week-old mice. Cyropreserved tissue also was stained with DNase I-594 for 45 minutes and then with DAPI. Actin immunolabeling was performed as described above on tissue sections of thickness 5 μm that were isolated from 6-week-old BALB/c mice, fixed with 2% PFA for 2 hours, paraffin-embedded, de-paraffinized and incubated in citric acid buffer, pH 6.0 at 95°C for 20 minutes for antigen retrieval.

Image acquisition and analysis

All images were captured as stacks of ten confocal slices (1 μm steps on the z-axis) using a Zeiss Axiovert 200 microscope with a Yokogawa spinning disk (Stanford Photonics XR/Mega-10 ICCD and QED InVivo version 3.11.2 software). Image acquisition parameters were kept constant for each experiment. All confocal images displayed throughout the Results section represent the center slice of a 1 μm confocal stack.

For colocalization analysis, images were deconvolved initially under default, adaptive, blind PSF settings using AutoDeblur version X1.41.2 software (MediaCybernetics) and imported into Imaris 6.1.3 (Bitplane AG, Zurich, Switzerland). A surface mask was generated using automatic threshold detection as previously described (Costes et al., 2004) for Pol II- and BrdU-labeled sites to exclude sites with a diameter of less than 0.2 μm. The Costes colocalization coefficients (Costes et al., 2004) were measured using Imaris and used to assess the nonrandom staining intensity levels of NLS–β-actin and endogenous nuclear actin with respect to areas occupied by Pol II and FUS masks, respectively. Briefly, all pixels contributing in a linear correlation between red and green fluorescence signals were automatically identified within each cell. To estimate random overlap, one channel was spatially randomized, and the resulting correlation between the two channels re-evaluated. By repeating randomization enough times, the probability of having random overlap for a given amount of correlation could be estimated (Costes et al., 2004). Using this method, all reported correlation values in this study are statistically significant (i.e. P<0.05).

To quantify cytoplasmic and nuclear YFP and YFP–β-actin levels, nuclear segmentation was performed on DAPI-stained images as described above, and YFP–positive cells were manually assigned nuclear coordinates. A threshold was applied.
manually for the YFP signal in each field, and the resulting mask was subtracted from the nuclear mask in order to delineate the YFP signal in the cytoplasm from that in the nucleus. The total intensity in this mask was then divided by the number of YFP-expressing nuclei, resulting in an average total cytoplasmic intensity per transfected cell for each imaged field. Cytoplasmic and nuclear YFP signal-intensity values between treated and untreated cells were compared using the Student’s t-test, and changes were reported as fold differences.

Quantification of the nuclear and cytoplasmic DNase I fluorescence intensity in S1 cells cultured on 2-D plastic and 3-D Matrigel was accomplished first by defining the cytoplasm was obtained using automatic threshold (isodata) on the actin channel. In order to assign the cytoplasm for each cell, we used the center of each segmented nucleus as a seed with different label values. All seeds were then expanded simultaneously by filling the actin mask with a watershed algorithm. This allowed the identification of each cytoplasm corresponding to the different segmented nuclei.

For cells transiently transfected with NLS- or NLS–β-actin, YFP–LAP was coexpressed as a nuclear marker and used to locate NLS- and NLS–β-actin-expressing positive cells. A threshold was applied to the YFP–LAP signal in order to eliminate background. This threshold was set manually as YFP–LAP expression levels varied considerably between cells. Nuclear segmentation of NLS- or NLS–β-actin-transfected cells was obtained by overlapping masks generated from both the DAPI and YFP channels. In each case, a threshold was set to eliminate non-overlapping regions.

To quantify FU incorporation or RNA Pol II levels in wild-type cells or cells treated with drugs, the center of each cell was defined by subtracting the actin channel from the DAPI channel. The nuclear area was then calculated by subtracting non-overlapping regions. The mean pixel intensity of the segmented nuclear area was normalized to the nuclear area. In addition, the mean pixel intensity of each nucleus was computed after subtracting the background pixels was normalized to the nuclear area. In addition, the mean pixel intensity of segmentation was performed initially using an in-house watershed-based segmentation.
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