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Comments
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Functional activity of RLIM/Rnf12 is regulated by phosphorylation-dependent nucleocyttoplasmic shuttling


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Abstract The X-linked gene Rnf12 encodes the ubiquitin ligase really interesting new gene (RING) finger LIM domain–interacting protein (RLIM)/RING finger protein 12 (Rnf12), which serves as a major sex-specific epigenetic regulator of female mouse nurturing tissues. Early during embryogenesis, RLIM/Rnf12 expressed from the maternal allele is crucial for the development of extraembryonic trophoblast cells. In contrast, in mammary glands of pregnant and lactating adult females RLIM/Rnf12 expressed from the paternal allele functions as a critical survival factor for milk-producing alveolar cells. Although RLIM/Rnf12 is detected mostly in lactating adult females RLIM/Rnf12 expressed from the paternal allele serves as a major sex-specific epigenetic regulator of female mouse nurturing tissues. Analysis of a conditional mutation of female nurturing tissues in mice. Expression of Rnf12 is regulated by phosphorylation-dependent nucleocytoplasmic shuttling of RLIM/Rnf12 coordinates cellular compartments during mammary alveolar cell survival.

INTRODUCTION

Really interesting new gene (RING) finger LIM domain–interacting protein (RLIM)/RING finger protein 12 (Rnf12) and Rnf6 define a class of RING finger ubiquitin ligases that is widely expressed during mouse embryogenesis (Tursun et al., 2005; Ostendorff et al., 2006). Whereas Rnf6 is detected in the nucleus and cytoplasm in cells (Tursun et al., 2005; Xu et al., 2009), RLIM/Rnf12 is detected mainly in the nuclear compartment, where it serves as a cofactor to promote or inhibit transcription factor activity in a context-dependent manner (Bach et al., 1999; Johnsen et al., 2009). Via proteasomal targeting, RLIM/Rnf12 regulates cellular levels of various mostly nuclear proteins, including CLIM/Ld/Ldb, LMO, HDAC2, TRF1, Smad4, and Rex1 (Ostendorff et al., 2002; Kramer et al., 2003; Her and Chung, 2009; Gontan et al., 2012; Zhang et al., 2012), thereby affecting the formation of transcriptional multiprotein complexes (Gungor et al., 2007). Moreover, functions of RLIM in promoting cell migration in vitro have recently been reported (Huang et al., 2011). RLIM/Rnf12 is encoded by the X-linked gene Rnf12 (Ostendorff et al., 2000), which plays major roles in sex-specific epigenetic regulation of female nurturing tissues in mice. Analysis of a conditional

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examined their relevance with respect to cellular localization and function of RLIM/Rnf12 protein. Whereas RLIM/Rnf12 protein is abundantly detected in the nuclei of cells, we find efficient nucleocytoplasmic shuttling of this protein and provide strong evidence that this process is regulated by phosphorylation of S214. We show that shuttling is critically involved in at least some of RLIM’s biological activities, including cell migration and mammary alveolar cell survival.

**RESULTS**

**Nuclear and cytoplasmic localization of RLIM**

Known substrate proteins targeted for proteasomal degradation by RLIM/Rnf12 are mainly localized in the nucleus. In agreement with published results that showed predominant nuclear localization of RLIM in cells in culture (Ostendorff et al., 2002) and in many cell types during mouse embryogenesis, including neurons (Tursun et al., 2005; Ostendorff et al., 2006), immunocytochemical experiments showed high levels of endogenous RLIM protein in nuclei of all cell lines investigated (αT3, CHO, Cos7, HeLa, HEK293T, MCF7, and MDA-MB-231; Figure 1A and data not shown). Using Western blots on nuclear and cytoplasmic fractions of αT3 cell extracts, however, we found a significant portion of cellular RLIM localized in the cytoplasm (Figure 1B). Examining cell types that exhibit cytoplasmic expression in vivo, we identified high levels of RLIM expression in the basal layer of human breast epidermis, where undifferentiated keratinocytes actively divide and which displayed significant RLIM staining in nuclear and cytoplasmic compartments (Figure 1C).

**RLIM shuttles between nucleus and cytoplasm**

Sequence comparison between RLIM and the closely related ubiquitin ligase Rnf6 revealed that both proteins display significant amino acid homologies over large parts of their sequence, including the RING finger, a basic domain (BD), and a leucine-zipper-like domain (L-Zip-L; Figure 2A). Furthermore, both proteins contained putative NLS and NES regions. However, whereas the putative NES was highly conserved between both proteins, the predicted NLS regions contained much less sequence homology (Figure 2A). We investigated the functionality of the putative NLS and NES in RLIM

**Nucleocytoplasmic shuttling can convey signals that coordinate crucial cellular processes, including gene expression, cell motility, and survival (Hervy et al., 2006). Because RLIM/Rnf12 contains putative nuclear localization sequences (NLS) and nuclear export sequences (NES), to illuminate mechanisms controlling RLIM, we investigated their relevance with respect to cellular localization and function of RLIM/Rnf12 protein. Whereas RLIM/Rnf12 protein is abundantly detected in the nuclei of cells, we find efficient nucleocytoplasmic shuttling of this protein and provide strong evidence that this process is regulated by phosphorylation of S214. We show that shuttling is critically involved in at least some of RLIM’s biological activities, including cell migration and mammary alveolar cell survival.**

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and Rnf6 by generating NLS–green fluorescent protein (GFP) fusion proteins. Whereas transfected GFP protein localizes to both the cytoplasm and nucleus, fusing the NLS domain of RLIM or Rnf6 to GFP resulted in strictly nuclear localization of the respective NLS-GFP fusion proteins (GFP-NLSRLIM and GFP-NLSRnf6; Figure 2B). Similarly, the GFP-NESRLIM and GFP-NESRnf6 fusion proteins localized to the cytoplasm. Furthermore, an RLIM-deletion mutant protein lacking the putative NLS was strictly localized to the cytoplasm (Figure 2C). These results show that the NLS is required for the nuclear localization of RLIM and that the NLS and NES are functional, suggesting that RLIM shuttles between compartments. To address this question, we noted that the NES of RLIM is required for nuclear localization. Staining of Myc-tagged full-length or mutant protein deleted of the putative NLS domain (Myc-RLIMΔNLS) was observed in HeLa cells and stained with anti-Myc antibodies (green). Nuclei were stained with Toto 3 dye (blue), and the cytoplasm was stained for actin with rhodamine–phalloidin (red). (D) RLIM shuttles between the nucleus and cytoplasm in heterokaryon assays. Human HeLa cells were transfected with Myc-RLIM or Myc-RLIMΔNES lacking the NES (white arrows) and fused with untransfected mouse NIH3T3 cells (red arrows). Arrows in PEG-treated cells point at nuclei in heterokaryons (circled). Note Myc-RLIM in the nuclei of NIH3T3 cells in a heterokaryon, indicating protein shuttling, whereas Myc-RLIMΔNES is retained in the nuclei of transfected HeLa cells. (E) The unique NLS of RLIM confers nuclear localization of RLIM. Cells transfected with a Myc-Rnf6 construct in which the NLS was replaced with that of RLIM (Myc-Rnf6ΔNLSNLSRLIM) and a Myc-RLIM construct in which the NLS was replaced with that of Rnf6 (Myc-RLIMΔNLSNLSRnf6) or SV40 (Myc-RLIMΔNLSNLSSV40). Cells were stained with anti-Myc antibody (green), Toto 3 dye (blue), and rhodamine-phalloidin (red).
was observed as early as 20 min, indicating rapid RLIM shuttling. We next examined protein localization in which the endogenous NLS was replaced in the context of the full-length protein. Replacing the endogenous NLS from Rnf6 with that of RLIM generated a protein Rnf6ΔNLS<sub>RLIM</sub> that localized to the nucleus, whereas replacing the NLS in RLIM with the one of Rnf6 (RLIMΔNLS<sub>Rnf6</sub>) was not sufficient for nuclear localization and targeted the resulting protein to the cytoplasm (Figure 2E). In addition, replacement of the endogenous NLS in RLIM with the NLS from simian virus 40 (SV40) failed to target the resulting protein to the nucleus. The functionality of the SV40 NLS was verified in GFP fusion experiments (data not shown). A similar replacement of NES domains in the context of full-length protein did not lead to a change in the localization of the proteins RLIMΔNES<sub>Rnf6</sub> and Rnf6ΔNES<sub>RLIM</sub> (data not shown). Taken together, these results demonstrate the nucleocytoplasmic shuttling of RLIM in cells and show that the NLS in RLIM is uniquely responsible for its nuclear localization and function.

**The NLS of RLIM is phosphorylated in conserved RSRSP domains**

Because ubiquitin ligases are often regulated via posttranslational modifications such as phosphorylation (Pickart, 2001; Bach and Ostendorf, 2003), we examined whether RLIM is phosphorylated in cells. Indeed, immunoprecipitation of endogenous RLIM from cell extracts of metabolically labeled cells using specific antibodies directed against GST revealed that RLIM is phosphorylated in cells (Figure 3A). Mapping experiments showed that RLIM full-length and RLIM mutants RLIM-M (208–403) and RLIM-MA (208–305), containing the C-terminal portion of RLIM, including the RING finger, are all phosphorylated (Figure 3A).

Owing to the very high degree of conservation of the NLS and secondary structure among different vertebrate species and the high frequency of sites for arginine- and/or proline-directed serine/threonine kinases within two RSRSP repeats (Figure 3B), we sought to determine whether these motifs are targets of phosphorylation and what functional role they might play in the regulation of RLIM. This was initially tested in vitro using purified glutathione S-transferase (GST) fusion proteins of the wild-type RLIM-NLS (amino acids 205–230), which contains all four serines (SSSS) as substrates for a kinase-enriched protein fraction from Hs578T human breast cancer cells. Of importance, mutation of all four serine residues to alanine (205-230-AAAA) resulted in complete inhibition of RLIM-NLS phosphorylation in vitro (Figure 3C). RLIM-NLS mutants containing the individual serine residues displayed strong phosphorylation of Ser-214 (205-230-ASAA), containing the C-terminal portion of RLIM, including the RING finger, are all phosphorylated (Figure 3A).
vitro kinase assays, we transfected expression vectors for myc-epitope–tagged fusion proteins of the various RLIM-NLS mutant proteins into HEK293 cells and tested their phosphorylation by metabolic labeling. As shown in Figure 3D and similar to the in vitro kinase data, Ser-214 (205-230-ASAA) and Ser-229 (205-230-AAAS) in the RLIM-NLS are highly phosphorylated in cells. These data identify RLIM as a protein phosphorylated in cells at many sites, including the NLS.

Phosphorylation at S214 occurs during differentiation of human epithelial tissues
It has been shown that CLIM cofactors are widely expressed in epithelial tissues, in particular in breast epithelia, where, in conjunction with LMO4, they are believed to contribute to mammary gland differentiation and the occurrence of breast cancer (Visvader et al., 2001; Wang et al., 2004). Given that CLIM cofactors and LMOs serve as substrate proteins for RLIM-mediated ubiquitinations (Ostendorff et al., 2002) and RLIM is expressed in breast epithelial cells and human estrogen receptor–positive breast cancer (Johnsen et al., 2009), we further examined RLIM expression in human epithelial tissues and tested whether S214 in RLIM is phosphorylated in vivo. To test this, we generated a polyclonal phospho-specific antibody recognizing phosphorylated S214 (RLIM-pS214) in the RLIM-NLS (205–230) and, as a control, phosphorylation-independent antibodies recognizing the same region of RLIM. The specificity of the RLIM-pS214 antibody was demonstrated using competition experiments in immunohistochemical stainings. We hybridized sections of human microinvasive squamous cell carcinomas or mammary gland with RLIM-pS214 and the phosphorylation-independent antiserum, adding excess amounts of synthetic RLIM-NLS peptides containing a phosphorylated S214 (RLIM-NLS-205-230-pS214) or an unphosphorylated RLIM-NLS peptide (RLIM-NLS-205-230). Whereas the signals of the non–phospho-specific antiserum were inhibited in the presence of RLIM-NLS-205-230-pS214 and RLIM-NLS-205-230 peptide (Supplemental Figure S2A), signals obtained by the phospho-specific RLIM-pS214 antibody were competed by adding excess amounts of the phosphorylated but not the unphosphorylated peptide (Figure S2, B and C), confirming the specificity of the antibodies.

Based on the use of a non–phospho-specific RLIM antibody on HFK cells, our results showed that in undifferentiated HFK cells a significant portion of RLIM localizes in the cytoplasm, whereas in differentiated cells RLIM mainly localizes in the nucleus (Figure 1D). To find the cellular compartment(s) in which the S214-phosphorylated RLIM localizes, we first performed immunocytochemistry using our antisera on HFK cells. In contrast to the non–phospho-specific antibody, we performed immunocytochemistry using our anti-sera on HFK cells. In contrast to the non–phospho-specific antibody, we detected exclusively nuclear RLIM staining using the RLIM-pS214 antibodies in both undifferentiated and differentiated cells (Figure 4A), consistent with a role of S214 phosphorylation for nuclear localization. Similarly, in HEK293 cells we also detected strictly nuclear signals using the phospho-specific antibody, whereas the phosphorylation-independent antibody yielded weak but detectable staining in the cytoplasm of cells (Supplemental Figure S2D). Next we tested RLIM S214 phosphorylation in human breast epidermis and human oral cavity epithelium specimens. The basal epithelia layer of normal breast epidermis showed strong...
staining using the phosphorylation-independent RLIM antibodies, whereas the phospho-specific RLIM-pS214 antibodies showed less staining of this layer (Figure 4B). In addition, whereas the RLIM-pS214 antibody showed only nuclear staining, we also observed cytoplasmic staining in cells of the basal layer using the phosphorylation-independent RLIM antibody (Figures 1C and 4B), corroborating results obtained in HFK cells (compare with Figures 1D and 4A). Similarly, in stainings of human oral cavity epithelium using the phosphorylation-independent RLIM antibody, we observed strong RLIM expression in oral epithelia, including basal layers (Figure 4C). Staining a parallel section with the phospho-specific RLIM-pS214 antibody, however, revealed significantly less staining in the basal layer, whereas suprabasal layers that contain differentiating keratinocytes displayed signal strengths similar to those obtained with the phosphorylation-independent RLIM antibody. Combined, these results indicate that pS214 phosphorylation occurs during the differentiation of epithelial tissues.

Phosphorylation at S214 is required for nuclear localization
We first examined whether NLS phosphorylation affects the cellular localization of RLIM, using NLS mutant proteins in the context of the isolated NLS. Comparing the localization of the wild-type GFP-NLS fusion protein with a GFP-NLS fusion protein in which all four serine residues in the NLS were replaced with alanine (AAAA) showed more cytoplasmic localization for the AAAA mutant protein, indicating that serine residues in the NLS are involved in the regulation of nuclear localization (Supplemental Figure S1B). We next generated several Ser-to-Ala mutations in the context of the full-length RLIM protein and tested their distribution in transfected cells. Results of these experiments showed that the S214 is required for nuclear localization, as the S214A mutant protein (SASS) localized mainly in the cytoplasm, whereas mutating the analogous serine (S229) in the second RSRSP motif (SSSA) had little effect (Figure 5). Conversely, nuclear localization was partially restored when only S214 was present (ASAA) when compared with the AAAA mutant, which localized mainly in the cytoplasm. Efficient nuclear localization, similar to SSSS-wild type, was detected in the ASAS mutant protein, which contained both S214 and S229. A mutant RLIM protein in which the serine residues were replaced by glutamic acid (EEEE) to mimic phosphorylation showed a strong bias toward nuclear localization. In this context, mutating both prolines in the RSRSP motifs to alanines mildly decreased the nuclear localization, and, not surprisingly, removing the first RSRSP motif (A211–215) completely abolished nuclear localization of mutant proteins (data not shown). Combined, these results show that S214 of RLIM is most crucial for nuclear localization, and other residues in the RSRSP motifs play additional roles.

Nucleocytoplasmic shuttling regulates the functional activity of RLIM/Rnf12
We first investigated the functional importance of RLIM nucleocytoplasmic shuttling for cell migration. Indeed, in agreement with previously published results (Huang et al., 2011), knocking down endogenous RLIM levels in MCF7 cells using two different short hairpin RNAs caused cells to migrate significantly slower in Transwell migration assays (Figure 6A), and knocking down RLIM in MDA-MB-231 breast cancer cells via small interfering RNAs (siRNAs) yielded similar results (Supplemental Figure S3). To examine the importance of RLIM localization for its ability to promote cell migration, we tested the migration potential of MCF7 cell lines that stably expressed RLIM mutant proteins in the context of the full-length protein via lentiviral infections. Whereas overexpression of wild-type (SSSS) and

EEEE proteins promoted cell migration, the NES-deleted RLIM or NLS mutant SASS and AAAA proteins were no longer able to enhance cell migration (Figure 6B). Expression of Myc-tagged wild-type and mutant proteins was verified in Western blots. To better assess and compare cellular levels of endogenous and induced exogenous RLIM, we added increasing concentrations of the proteasome inhibitor lactacystin. Because the ΔNES and both NLS mutants, which locate in the nucleus (Figure 3D) and cytoplasm (Figure 6A), respectively, fail to promote cell migration, these results link protein shuttling with cell migration.
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infected with lentivirus expressing the shuttling-deficient RLIM mutants or, as control, wild-type RLIM. Indeed, whereas the number of cells did not change upon lentiviral expression of GFP or a Myc tag alone when compared with uninfected cells, physiological levels of exogenous Myc-RLIM (SSSS) resulted in significantly increased cell numbers (Figure 7D). Because RLIM expression does not affect proliferation (Jiao et al., 2012), these results reflect a rescue from apoptosis. The shuttling-deficient Myc-SASS or Myc-ΔNES mutant RLIM, however, displayed a markedly lower rescue potential than wild-type RLIM (Figure 7D and Supplemental Figure S4). Combined, these results indicate an important contribution of nucleocytoplasmic shuttling of RLIM in its ability to promote alveolar cell survival.

DISCUSSION

In female mice, RLIM/Rnf12 appears to play essential, sex-specific roles in developmental processes such as XCI (Shin et al., 2010) and mammary alveolar cell survival and involution (Jiao et al., 2012). Other roles that RLIM might play are nonessential in mice because male mice carrying a germline Rnf12 KO appear healthy and are fertile (Shin et al., 2010). Thus, because RLIM is widely expressed in many epithelial and nonepithelial tissues in mouse embryos and adult animals (Bach et al., 1999; Ostendorff et al., 2000, 2006), the biological importance of RLIM in most of these tissues is unclear. Nevertheless, in cells RLIM reportedly contributes to apparently nonessential cellular functions such as cell migration (Huang et al., 2011), adjustment of levels of transcription factors/cofactors, assembly of multiprotein complexes, and transcriptional activity of several.

We next investigated the importance of RLIM S214 phosphorylation and nucleocytoplasmic shuttling for its functions in mammary glands. Hybridizing sections with phospho-specific RLIM-pS214 and phosphorylation-independent RLIM antibodies revealed that, similar to other epithelial cell types (Figure 4), RLIM is phosphorylated in nuclei of mammary epithelial cells (Figure 7A). Immunocytochemical costaining of primary mammary epithelial cells showed that RLIM is localized in both nucleus and cytoplasm of basal CK14-positive and luminal CK18-positive cell types (Figure 7B). Moreover, RLIM costainings with rhodamine–phalloidin revealed that in the cytoplasm RLIM colocalizes with actin filaments (Figure 7C). This was confirmed in costainings using actin antibodies (data not shown), suggesting that RLIM shuttling occurs between sites on the actin cytoskeleton and the nucleus. Using a conditional Rnf12-KO mouse model, we recently reported a crucial function of RLIM in the survival of mammary milk-producing alveolar cells (Jiao et al., 2012). Indeed, alveolar cells lacking RLIM undergo apoptosis when differentiated in culture, and cell death can be partially rescued by expressing exogenous RLIM via lentiviral infections. Thus, because these cells require RLIM expression for their survival, we tested the rescue potential of exogenous RLIM shuttling mutants in primary mammary epithelial cells lacking RLIM. To carry out these experiments, we isolated primary mammary epithelial cells of adult virgin female mice in which the floxed Rnf12 alleles are targeted to the mammary gland using transgenic mice that express Cre recombinase (Cre) under the control of the mouse mammary tumor virus (MMTV) long terminal repeat (Wagner et al., 1997; Jiao et al., 2012; Rnf12fl/fl x MMTV-Cre). Cells were then differentiated in culture and infected with lentivirus expressing the shuttling-deficient RLIM mutants or, as control, wild-type RLIM. Indeed, whereas the number of cells did not change upon lentiviral expression of GFP or a Myc tag alone when compared with uninfected cells, physiological levels of exogenous Myc-RLIM (SSSS) resulted in significantly increased cell numbers (Figure 7D). Because RLIM expression does not affect proliferation (Jiao et al., 2012), these results reflect a rescue from apoptosis. The shuttling-deficient Myc-SASS or Myc-ΔNES mutant RLIM, however, displayed a markedly lower rescue potential than wild-type RLIM (Figure 7D and Supplemental Figure S4). Combined, these results indicate an important contribution of nucleocytoplasmic shuttling of RLIM in its ability to promote alveolar cell survival.
classes of transcription factors (Bach et al., 1999; Ostendorff et al., 2002; Gungor et al., 2007; Johnsen et al., 2009). Our finding that this protein is localized in both nucleus and cytoplasm in cells and shuttles between these cellular compartments is likely relevant for all of these functions.

RLIM phosphorylation
The NLS in RLIM containing two core RSRSP motifs is highly conserved among vertebrate species (Figure 3B). Secondary structural predictions place two α-helices in the NLS region, which are interrupted by the RSRSP motifs. A BLAST analysis of the RSRSP motif revealed many nuclear proteins that contain tandem RSRSP motifs (Supplemental Figure S5). Some of these proteins belong to the Ser/Arg-rich (SR) class of proteins known to contain many RS repeats (Graveley, 2000), whereas many others are not considered SR proteins. Of interest, nucleocytoplasmic shuttling of several of these proteins has been reported. The RNA-binding protein 1 (Lykke-Andersen et al., 2001) and the SR protein SF2/ASF both shuttle between the cytoplasm and nucleus, and, similar to RLIM, this shuttling is phosphorylation dependent for SF2 (Sanford et al., 2005). Thus the RSRSP motifs likely serve as a prototypic sequence for phosphorylation-dependent nuclear localization. Because Rnf6 contains functional NLS and NES (Figure 2B), it is likely that these also mediate nucleocytoplasmic shuttling. However, the RLIM NLS is unrelated to that of Rnf6, indicating divergent mechanisms that regulate protein shuttling.

In most tissues during mouse development (Ostendorff et al., 2006) and in all cell lines tested, cellular RLIM levels appear highest in the nucleus, where it is phosphorylated at S214. This argues that the kinase responsible for the NLS phosphorylation is widely expressed and/or that many different kinases may phosphorylate the RSRSP motif. Indeed, >50 kinases are predicted to phosphorylate the S214 by the PhosphoNET kinase predictor (www.phosphonet.ca), and phosphorylation was confirmed in human RLIM using quantitative phosphoproteomics (Olsen et al., 2010). This, combined with the finding that S214 phosphorylation is required for nuclear import, suggests that RLIM is phosphorylated at S214 in the cytoplasm, and this phosphorylation event triggers efficient binding to import proteins/nuclear import. However, the exact mechanisms regulating S214 phosphorylation and dephosphorylation and the nuclear import mechanisms remain unclear.

Our results show that besides the RSRSP motifs in the NLS, RLIM is phosphorylated at several different sites in cells (Figure 3A), suggesting that activities of RLIM other than its cellular localization also may be regulated by posttranslational phosphorylation. Indeed, a common theme emerging in recent years is that the activity of many ubiquitin ligases is regulated via posttranslational modifications, including phosphorylation (Hunter, 2007).

FIGURE 7: Nucleocytoplasmic shuttling regulates RLIM’s ability to act as alveolar survival factor. (A) RLIM is phosphorylated in nuclei of mammary epithelial cells. Immunohistochemical sections of adult human mammary gland were stained with non-phospho-specific antibodies recognizing RLIM (left) or phospho-specific peptide RLIM-pS214 antibodies (right). Scale bars, 25 μm. (B) Dual localization of RLIM in the nucleus and cytoplasm in basal and luminal primary mammary cells. Primary mammary epithelial cells were isolated from an adult wild-type virgin female mouse and costained with antibodies directed against RLIM and basal marker CK14 or luminal marker CK18. (C) RLIM is associated with the actin cytoskeleton in primary mammary cells. Primary mammary epithelial cells were isolated from an adult wild-type virgin female mouse and costained with antibodies directed against RLIM and rhodamine–phalloidin. Boxed area is shown in higher magnification. (D) Primary mammary epithelial cells that lack RLIM were isolated from adult Rnf12<sup>fl/fl</sup> x MMTV-Cre virgin female mice. Cells were infected with lentivirus expressing Myc-tagged wild-type (SSSS) and shuttling-deficient RLIM mutant proteins (SASS) and ΔNES proteins. Cells infected with lentivirus expressing GFP and Myc-tag and uninfected cells were used as control. Averages ± SEM for four independent measurements.
Relevance of RLIM shuttling for its biological functions

In this article, we provide evidence that protein shuttling occurs between sites of cytosolic actin filaments and the nucleus. Indeed, we show important functions of RLIM shuttling for alveolar cell survival and cell migration. The process of shuttling appears to be of high importance to regulating these functions, as expression of wild-type protein promotes both functions, but expression of cytoplasmic or nuclear shuttling-deficient RLIM mutant protein is much less efficient in mediating these processes. These results indicate that RLIM serves as a signaling molecule, which carries signals between these compartments. The fact that cell migration is mediated by restructuring of the actin cytoskeleton with which RLIM is associated (Figure 7B) suggests that RLIM/Rnf12 exerts its function on proteins localized with the actin cytoskeleton that mediate this process. In this context it is interesting to note that the RLIM-related protein Rnf6 regulates local actin dynamics in axonal growth cones in neurons by targeting LIM kinase 1 (LIMK1) for proteasomal degradation (Tursun et al., 2005). However, although RLIM can interact with the LIM domain of LIMK1 (Bach et al., 1999) and mediate its ubiquitination in vitro (Tursun et al., 2005), our results obtained by Western blotting and immunostainings in cells depleted of RLIM indicate that cellular LIMK1 is not regulated by RLIM (data not shown). The finding that cytoplasmic and nuclear shuttling-deficient RLIM mutant proteins (RLIM-SASS and RLIMANES, respectively) are much less efficient in promoting cell migration and alveolar cell survival indicates that both cytoplasmic and nuclear functions of RLIM are important. Thus RLIM acts as a signaling molecule that synchronizes functions in cellular compartments, and our results provide strong evidence that this is important to carry out these functions. Indeed, dual cytoplasmic and nuclear distribution has been observed for several proteins, including cytoplasmic LIM proteins, which are known to shuttle and signal between cellular adhesion sites and the nucleus. Similar to RLIM, these proteins often act as transcriptional cofactors in the nucleus to modulate transcription factor activity (Bach, 2000; Kadrmas and Beckerle, 2004). In this context, it is interesting to note that RLIM is detected along the plasma membrane in cells located in the epidermal basal cell layer and to a lower extent in mammary cell types (Figures 1, C and D, and 7, B and C).

Besides its crucial role in alveolar cell survival, it has been suggested that RLIM triggers involution via targeted proteasomal degradation by wear-in-induced autoubiquitination (Jiao et al., 2012). This scenario would be analogous to the signal-induced apoptosis in thymocytes mediated by the RING finger ubiquitin ligase inhibitor of apoptosis (IAP), which also serves as a survival factor in this cell type. Of interest, IAP proteins localize to the cytoplasm, where they interact with a variety of proteins, including caspases, leading to their inhibition and thereby promoting cell survival (Vucic et al., 2011). Consistent with nucleocytoplasmic shuttling involved in RLIM's survival function, our results show RLIM localization in both compartments. Because RLIM rapidly shuttles (Figure 2D and data not shown) and RLIM levels in alveolar cells drop dramatically within 6 h upon forced weaning (Jiao et al., 2012), it is conceivable that to deplete cellular RLIM levels during involution it would be sufficient to target RLIM for degradation in only one of the compartments (e.g., the cytoplasm). Hence, to further decipher the role of RLIM in promoting alveolar cell survival and trigger involution, it will be interesting to elucidate the protein network with which RLIM is associated in the cytoplasm of mammary epithelial cells. Whether nucleocytoplasmic shuttling is important also for RLIM's functions during XCI (Jonkers et al., 2009; Shin et al., 2010; Barakat et al., 2011) is not clear. Because RLIM/Rnf12 does not appear enriched at inactive X chromosomes and X chromosomes that undergo XCI, however, the colocalization of RLIM with actin in the cytoplasm (Figure 7B and data not shown) is intriguing, as actin exists also in the nucleus and may therefore be involved in the nuclear distribution of RLIM. Indeed, nuclear β-actin/actin-related proteins participate in chromatin-remodeling and histone acetyltransferase complexes, thereby influencing long-range chromatin organization (Pederson, 2008; Visa and Percipalle, 2010). Moreover, the two highly conserved core RSRSR motifs are also found in the zebrafish RLIM/Rnf12, and functions of this ubiquitin ligase during embryonic stem cell fate have been reported via proteasomal targeting of Smad4, which is localized in both cytoplasmic and nuclear compartments (Zhang et al., 2012). Thus the regulated nucleocytoplasmic shuttling of RLIM is likely to have important developmental functions in this organism.

In summary, we propose that phosphorylation-dependent nucleocytoplasmic shuttling of RLIM functionally coordinates cellular compartments during cell migration and for the survival of alveolar cells in the mammary gland.

MATERIALS AND METHODS

Plasmids/lentiviral constructs

Plasmids used in this work were RLIMANLS (Δ206–226) in pCS2MT and pGEX-KGK, RLIMANES (Δ502–513) in pCS2-MT and pGEX-KGK, GFP-NLSαL6 (186–208) in pEGFP-C3 (Clontech), GFP-NESαL6 (566–577) in pEGFP-C3, GFP-NLSRLIM (206–226) in pEGFP-C3, GFP-NESRLIM (501–513) in pEGFP-C3, pCS2-MT-Rnf6ΔRING (Δ589–667), pCS2-MT-Rnf6ΔARINGANES (Δ566–577; Δ589–667), pCS2-MT-RLIMΔRINGANLS (Δ206–226; Δ544–600), and previously described cDNA clones RLIMΔRING, N-RLIM, M-RLIM, and C-RLIM in euakaryotic and bacterial expression vectors (Bach et al., 1997, 1999; Ostendorff et al., 2002). RLIM serine point mutations were generated in the context of the pCS2-MT expression plasmid. Lentiviral infections were performed using lentiviral vectors (pLenti CMV/TO Puro) expressing NLS/NES mutant RLIM as described (Campeau et al., 2009). Correct sequence was verified via DNA sequencing.

Antibodies

Polyclonal antisera raised in rabbit and guinea pig against mouse RLIM and Lhx3 have been described (Ostendorff et al., 2002, 2006). Phospho-specific and phosphorylation-independent S214-RLIM antibodies (α-RLIM-pS214 and NP-8043, respectively) were generated and verified by enzyme-linked immunosorbent assay by Biogenes (Berlin, Germany). Anti-Myc polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal 9E10 (Roche, Mannheim, Germany) antibodies and anti-HA.11 monoclonal antibody (BABCO, Richmond, CA) were used to detect/immunoprecipitate Myc- and hemagglutinin (HA)-tagged proteins, respectively. CK14 and CK18 antibodies (Abcam, Cambridge, MA) were used in immunostainings, and anti-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Sigma-Aldrich (St. Louis, MO) and Chemicon (Temecula, CA), respectively. Secondary antibodies were horseradish peroxidase–anti-mouse and horseradish peroxidase–protein A (Bio-Rad, Munich, Germany). Biotinylated secondary antibodies for the immunohistochemistry were goat anti-guinea pig (Vector Laboratories, Burlingame, CA) and goat anti-rat (MoBiTec, Göttingen, Germany). For immunocytochemistry we used Alexa 488 goat anti-mouse (MoBiTec), Alexa 488 goat anti–guinea pig (MoBiTec), Cy3 goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA), Cy5 goat anti-rabbit (MoBiTec), and Cy5 goat anti-rat (MoBiTec). Rhodamine–phalloidin and Toto-3 dye (Molecular Probes, Eugene, OR) were used to visualize actin filaments and nuclei, respectively.

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Cell lines used in this work were CHO, Cos-7, HEK293T, HeLa, Hs578T, MCF7, MDA-MB-231, NIH3T3, and the pituitary gonadotrope cell line αT3 (Windle et al., 1990). HeLa and Hs578T cells were grown in DMEM or DMEM/F12, respectively (PAA Laboratories, Pasching, Austria), containing 10% (vol/vol) fetal bovine serum (FBS; PAA Laboratories) and 1× antibiotic-antimycotic solution (Invitrogen). Cells were seeded in 24-well or 10-cm plates and transfected at 80% confluence with various combinations of plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. Transient transfections, cotransfections, and immunocytochemical experiments were carried out as described (Ostendorff et al., 2002; Tursun et al., 2005). Cells were treated with leptomycin B (a kind gift of M. Yoshida, University of Tokyo) as reported previously (Kudo et al., 1999). The heterokaryon shutting assay was performed essentially as described (Katahira et al., 1999). Briefly, HeLa cells expressing Myc-RLIM or Myc-RLIMANES were cocultured with NIH3T3 cells on coverslips for 4 h. After treatment with 10 μM cycloheximide for 30 min, the cells were fused by 50% PEG 1500 and incubated in the presence of cycloheximide for 1 h. The cells were fixed, permeabilized, and incubated with anti-Myc monoclonal antibody, followed by incubation with Alexa 488-labeled anti-mouse immunoglobulin G. HFK cells (a kind gift of E. Androphy, University of Massachusetts Medical School) were cultured in serum-free keratinocyte media (Gibco/Life Technologies, Carlsbad, CA) supplemented with epidermal growth factor and pituitary extract. Cells were differentiated by adding fetal calf serum to 10% final concentration. For transient transfections of siRNA, cells were transfected twice in a 12-h period using FuGENE (Roche) according to the manufacturer's instructions. siRNAs against human RLIM have been described (Ostendorff et al., 2002).

Western blotting

Cell extracts were harvested in RIPA buffer (phosphate-buffered saline, 1% [wt/vol] Nonidet P-40, 0.5% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] SDS) containing 100 μg/ml phenylmethylsulfonyl fluoride (PMFSF), 2 μg/ml aprotinin, 10 μg/ml leupeptin, and 500 μM sodium orthovanadate. Protein was separated on a SDS–10% (wt/vol) polyacrylamide gel and blotted onto Protran nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Primary antibodies were detected by enhanced chemiluminescence (GE Healthcare Life Sciences, Piscataway, NJ) using horseradish peroxidase–conjugated antibody A (Bio-Rad).

In vitro kinase assays

A cellular fraction enriched for RLIM-NLS kinase activity was obtained by performing a 40–60% ammonium sulfate precipitation. Briefly, Hs578T breast cancer cells were grown to confluence and harvested in RIPA buffer containing protease and phosphatase inhibitors. Cell lysate was initially precipitated by adding saturated ammonium sulfate to a concentration of 40% (vol/vol), and the insoluble fraction was removed by centrifugation. After centrifugation, the active fraction was obtained by further centrifugation after an increase in the ammonium sulfate concentration to 60% (vol/vol). This fraction was then resuspended in kinase buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 10 mM MgCl₂, 25 mM NaCl, 10 mM sodium β-glycerophosphate, 0.1 mM Na₂VO₃, 0.2 mM PMFSF, 1 mM dithiothreitol, 10 μM ATP), aliquoted, and stored at −80°C until use in in vitro kinase assays. For in vitro kinase assays 1 μg of GST fusion protein was incubated with 1 µl of kinase-enriched Hs578T cell extract in a total volume of 30 μl of kinase buffer containing 1 μCi of [³²P]γ-ATP (220 TBq/mmol). After 20 min of incubation at 30°C, Laemmli buffer was added and samples were boiled for 5 min, separated by SDS–PAGE, and blotted onto nitrocellulose membranes. The blots were then analyzed by autoradiography for the presence of phosphorylated proteins. Equal loading of GST fusion proteins was demonstrated by anti-GST Western blot analysis.

Metabolic labeling

HEK293 cells were grown in 6-cm plates and transfected with 10 μg of myc-fusion protein expression plasmids using the polyethylenimine procedure. Briefly, 10 μg of DNA was incubated with 500 μl of PBS containing 100 μM polyethylenamine. After 20 min of incubation at room temperature, transfection mixes were added to cells and incubated overnight. At 16 h after transfection, cells were incubated in serum-free/phosphate-free medium for 30 min and another 4 h in 2.5 ml of phosphate-free medium containing 0.25 μCi of [³²P]orthophosphate. Protein was harvested in 1 ml of RIPA buffer containing phosphatase and protease inhibitors. Myc-fusion proteins were recovered by immunoprecipitation overnight with 1.5 μg of anti-myc 9E10 mouse monoclonal antibody (Roche). Immune complexes were recovered with Protein A/G Agarose (Santa Cruz Biotechnology), washed three times with RIPA buffer containing phosphatase and protease inhibitors, boiled in Laemml buffer, subjected to SDS–PAGE analysis, and blotted onto nitrocellulose membranes. The blots were then analyzed by autoradiography for the presence of phosphorylated proteins. Equal expression and loading of myc-fusion proteins was demonstrated by anti-myc Western blot analysis with a polyclonal anti-myc antibody. Phosphorylation of endogenous RLIM protein was similarly investigated using a specific anti-RLIM monoclonal antibody and metabolically labeled protein extracts from MDA-MB-231 breast cancer cells, which express high levels of RLIM protein.

Human tissue samples and immunohistochemistry

The paraffin-embedded primary breast and head and neck cancer tissue specimens were obtained from the Institute of Pathology, University Medical Center Hamburg-Eppendorf, in an anonymous manner. Sections from Formalin-fixed, paraffin-embedded tissue were deparaffinized and subsequently subjected to heat pretreatment in antigen decloaker chamber (Decloaker; Biocarta, Hamburg, Germany) for 5 min at 120°C. After blocking of endogenous peroxidase activity, RLIM expression was visualized by using polyclonal rabbit antibodies NP-8043 (non–phospho-specific) antibody (1:500 dilution, 1 μg/ml), α-RLIM-pS214 (1:250 dilution, 1.3 μg/ml), and α-RLIM (1:1000 dilution; Ostendorff et al., 2002). Slides were incubated with the primary antibodies for 16–18 h at 4°C in a humidified chamber. Application of the primary antibody was followed by incubation with biotinylated goat anti-mouse/anti-rabbit immunoglobulins and streptavidin conjugated to horseradish peroxidase (ChemMate Detection Kit, Peroxidase/AB, Rabbit/Mouse; DakoCytomation, Hamburg, Germany). 3,3′-Diaminobenzidine tetrahydrochloride chromogen solution and a substrate buffer containing hydrogen peroxide served as substrate system. Sections were counterstained with Mayer’s hemalaun solution (Merck, Darmstadt, Germany) and permanently mounted.

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Cell migration assays

Cell migration assays were performed essentially as described (Chen et al., 2007). Briefly, cells were rinsed in serum-free media and plated into migration chambers (BD Biosciences, San Diego, CA) at a density of $5 \times 10^4$ cells/insert. Media containing 10% FBS as a chemoattractant was placed in the lower chamber. After 20 h, cells were fixed, stained with Giemsa reagent, and counted at 10× magnification. Five fields were counted at this magnification for each migration insert and the sum of these fields determined. The migration activity of control cells was normalized to 1 and the data plotted relative to the controls.

Alveolar cell survival assays

Alveolar survival assays were performed on primary mammary epithelial cells prepared from adult virgin females lacking mammary RLIM/Rnf12 (Rnf12<sup>+/−</sup> x MMTV-Cre) essentially as described (Jiao et al., 2012). Briefly, fourth inguinal mammary glands were isolated from 2-mo-old virgin mice and digested using collagenase/hyaluronidase for 3-6 h. The suspension was incubated with trypsin/EDTA/DnaseI, followed by filtration through a 40-μm cell strainer. Cells were cultured overnight with differentiation medium (DMEM/10% FBS, 0.5% penicillin/streptomycin, and 1% sodium pyruvate) before lentiviral infections. Lentiviral infections of primary mammary epithelial cells were isolated and differentiated for 1 d in FBS before lentiviral infections. Lentiviral infections of primary cells or MCF7 cells were performed using lentiviral vectors (based on pLenti CMV/TO Puro) as described (Campeau et al., 2009) expressing GFP, 6xMyc tag (Myc), Myc-RLIM, and Myc-RLIM mutants SASS, EEEE, AAAA, and ANES. Two days after infections, cells were aliquoted and seeded on 96-well plates for counting using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI). Results were pooled from eight individual wells per measuring point. Three independent experiments were carried out. Wt and Rnf12 mutated mice (Jiao et al., 2012) were bred in the animal facility of the University of Massachusetts Medical School according to National Institutes of Health guidelines established by the Institute of Animal Care and Usage Committee.

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