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COMMENTARY

Regulation of RhoA activation and cytoskeletal organization by acetylation

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ABSTRACT

Rho GTPases regulate cell motility in a large part through control of actin cytoskeletal organization. The activation state of Rho proteins is regulated by a wide variety of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins that are differentially expressed among cell types and disease states. The RhoA specific GEF neuroepithelial transforming gene 1 (Net1) is highly expressed in many cancer cells and stimulates cell motility, invasion and cell spreading in response to a variety of ligands. A key feature of Net1 proteins is that they are sequestered in the nucleus in the absence of a motility stimulus. We have recently found that accumulation of the Net1A isoform outside the nucleus, which is the primary Net1 isoform controlling cell motility, is regulated by its acetylation status. Here, we describe acetylation as a novel mechanism of RhoGEF regulation in cell motility that can be targeted in cancer and metastasis.

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Introduction

Elucidation of mechanisms underlying of cell motility is fundamental to our understanding of cancer metastasis.[1,2] Cell motility requires the generation of a dynamic actin cytoskeleton, which is controlled by cyclic activation of Rho GTPases. The Rho GTPase family consists of 20 separate genes, with RhoA, Cdc42, and Rac1 being the most well studied.[3,4-6] Activation of classical Rho GTPases such as RhoA is controlled by guanine nucleotide exchange factors (GEFs), which catalyze GDP release to allow GTP binding.[7] Inactivation of RhoA is regulated by another family of proteins known as GTPase activating proteins (GAPs), which stimulate the intrinsic GTPase activity of Rho proteins up to 1000-fold.[8] Both the RhoGEF and RhoGAP families are quite large, with nearly 70 genes in each category.[7,8] Cells typically express multiple GEFs and GAPs that regulate different, often overlapping sets of Rho proteins. Thus, a daunting problem when considering cancer cell motility is identifying the particular GEFs or GAPs that might serve as nodal points which could be targeted for therapeutic intervention.

Regulatory mechanisms controlling the nuclear localization of Net1 isoforms

The neuroepithelial transforming gene 1 (Net1) is a RhoGEF that mediates cell motility and extracellular matrix invasion in multiple cancer cell types.[9-11,12] In cells it specifically activates RhoA and RhoB, but not the related GTPases Rac1 or Cdc42.[13,14] Two isoforms of Net1 are expressed in most cells, Net1 and Net1A, which differ only in their N-terminal regulatory domains.[15,16] Expression of Net1 isoforms is controlled by distinct promoters which are responsive to different ligands.[17] For example, estrogen primarily stimulates Net1 expression while TGFβ favors expression of Net1A.[12,17] Net1 proteins are unusual among RhoGEFs in that they localize to the nucleus in quiescent cells.[15,18] However, RhoA must be activated at the plasma membrane to stimulate cell motility, indicating that Net1 proteins must relocalize to the plasma membrane to promote RhoA activation and cell movement. Such mechanisms controlling Net1 isoform localization are only recently being uncovered.

Schmidt and Hall were the first to show that the longer Net1 isoform contained 2 nuclear localization signal (NLS) sequences in its unique N-terminal regulatory domain.[18] However, their analysis suggested that there were additional determinants of nuclear targeting, as deletion of both of these NLS sequences did not fully preclude nuclear localization of Net1. Moreover, mechanisms controlling the nuclear localization of Net1A were unknown. Using newer NLS sequence prediction programs and site directed mutagenesis, we were able to identify 2 additional NLS sequences in Net1 that were

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conserved in Net1A. Mutation of all 4 NLS sequences in Net1, or the 2 in Net1A, created proteins that were excluded from the nucleus.[16]

Acetylation of Net1A promotes RhoA signaling

Previously, we had shown that the motility of many invasive breast cancer cells largely depended on Net1A expression[10] and that Rac1 activation downstream of integrin engagement stimulated relocalization of Net1A but not Net1.[19] Therefore, we focused on understanding mechanisms controlling the cytosolic localization of Net1A.

To this end, we examined post-translational modifications that occurred upon co-expression of constitutively active Rac1. Using mass spectrometry, we were unable to find phosphorylation differences in Net1A stimulated by Rac1. We did surprisingly identify lysine 83 as being acetylated, and in a subsequent analysis we showed that treatment with the deacetylase inhibitor trichostatin A (TSA) resulted in acetylation of 5 sites within Net1A. Two of these were within or adjacent to the second NLS sequence within Net1A. As acetylation would nullify the positive charge required for effective contribution to an NLS sequence,[20] we reasoned that Net1A acetylation might inhibit the function of that NLS.

Similar to Rac1 activation, treatment of cells with Sirtuin and histone deaceytlase inhibitors (HDACs) also stimulated Net1A relocalization.[16] Using acetylation precluding and mimicking amino substitutions, we found that acetylation of the N-terminal sites rather than the DH domain accounted for Net1A relocalization in the cytosol after deacetylase inhibition.[16] Importantly, glutamine substitution of the N-terminal sites within Net1A led to an increase in RhoA activity, as might be expected to accompany Net1A relocalization outside the nucleus. These findings are unique as no other RhoGEFs have been shown to be regulated by site specific acetylation.

To understand the biological relevance of Net1A acetylation, we examined the effect of EGF on Net1A localization, as this growth factor often plays a pivotal role in controlling breast cancer cell motility and is known to activate Rac1.[16,19] We observed that EGF stimulated Net1A relocalization in a Rac1-dependent manner, similar to LPA or integrin ligation.[10,19] Using acetylation mutants of Net1A, we also showed that the N-terminal acetylation sites are indeed necessary for EGF-induced Net1A relocalization. Furthermore, expression of Net1A containing glutamine substitutions at these sites stimulated myosin phosphatase targeting protein 1 (MYPT1) and myosin light chain 2 (MLC2) phosphorylation, which are downstream effects of RhoA activation required for increasing actomyosin contraction. To corroborate these results, we examined F-actin accumulation and focal adhesion maturation, which are also robust readouts of RhoA signaling, in *Net1* knockout mouse embryonic fibroblasts in which acetylation-mimic or deficient Net1A mutants were expressed. Consistent with effects on RhoA activation, we found that acetylation of the N-terminal sites is also required for F-actin accumulation and focal adhesion maturation.

Overall our results suggest that lysine acetylation of Net1A is critical for its relocalization out of the nucleus

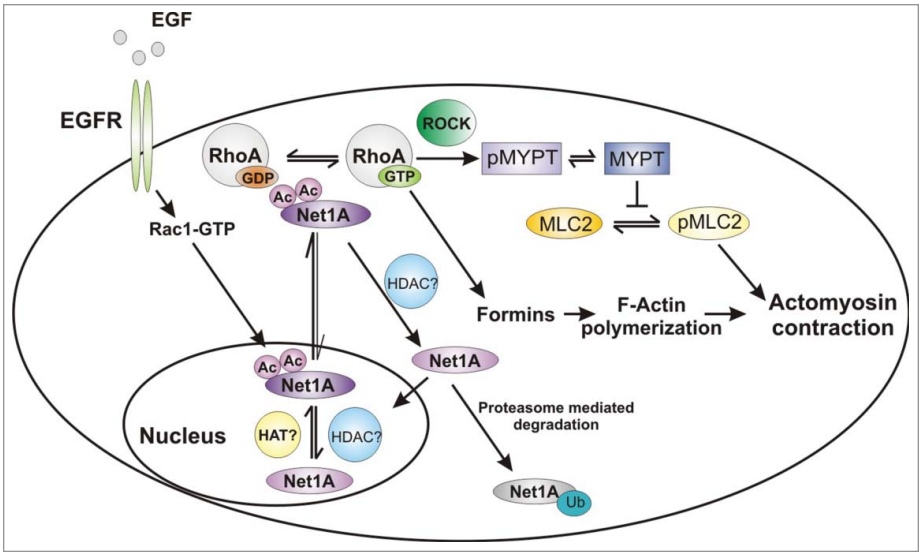

Figure 1. Model for the role of N-terminal acetylation of Net1A in cell motility.Net1A localizes to the nucleus in unstimulated cells. EGF-induced Rac1 activation or intrinsic Net1A N-terminal acetylation by HATs results in relocalization of Net1A in the cytosol. Acetylated Net1A then activates RhoA to promote MYPT1 inactivation and subsequent accumulation of phosphorylated MLC2, leading to actomyosin contraction. RhoA activation also stimulates formins, which catalyze F-actin polymerization.

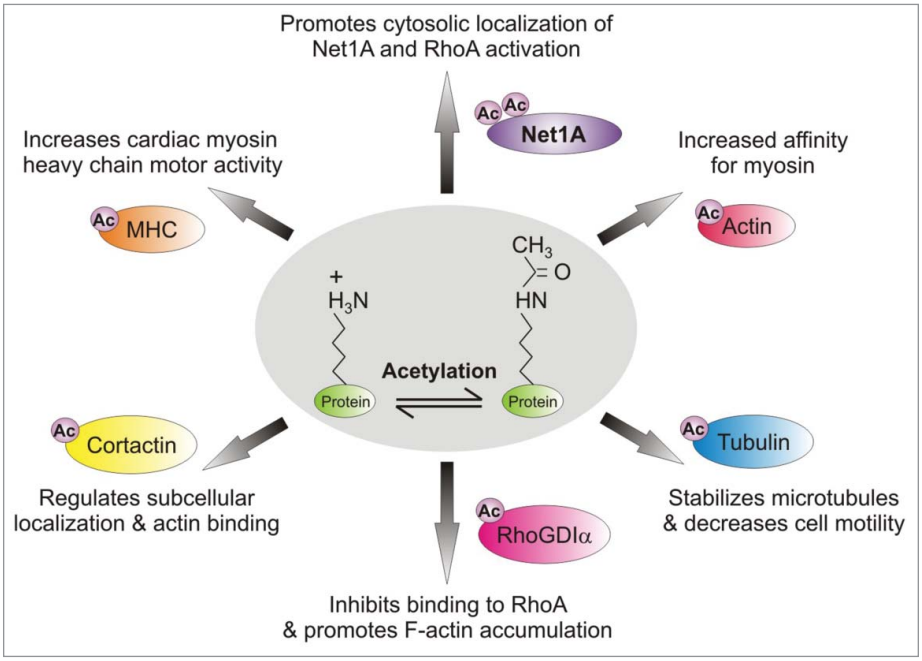

Figure 2. Acetylation of multiple proteins involved in actomyosin contraction and cell motility Acetylation alters the function and subcellular localization of proteins that regulate the actin and tubulin cytoskeletons.

and to promote RhoA signaling, F-actin accumulation and focal adhesion maturation. We expect that Net1A acetylation reduces the rate of re-import into the nucleus by inactivating one of its 2 NLS sequences. Acetylation may also protect Net1A from proteasome-mediated degradation, as we have shown previously that Net1A is ubiquitylated and that treatment with the proteasome inhibitor MG132 extends the length of time that Net1A resides outside the nucleus following integrin ligation (Fig. 1).[19,21]

Conclusions

Further work will be required to fully understand the regulatory mechanisms controlling Net1A subcellular localization. For example, the identity of the lysine acetyltransferases and deacetylases that control Net1A acetylation are unknown. In this regard, overexpression of the acetyltransferases p300, PCAF or CBP did not stimulate Net1A acetylation or relocalization. Similarly, overexpression of HDACs 2, 5, 6, or Sirtuins 2, 6 or 7 did not prevent Net1A cytoplasmic localization (Song and Frost, unpublished data). These findings are distinct from recently published effects on Rho guanine nucleotide dissociation inhibitor α (RhoGDIα), where it was observed that its acetylation can be stimulated by overexpression of PCAF, p300 and CBP and inhibited by overexpression of HDAC6 or SIRT2.[22]

It is noteworthy that we did not observe significant relocalization of the Net1 isoform following acetylation. This may be due to differences in the number of NLS sequences between isoforms, as Net1 contains 4 NLS sequences while Net1A only has 2.[16] Thus, Net1 might have a more robust interaction with nuclear importins as compared to Net1A, thereby reducing its residence time in the cytoplasm. Mechanisms controlling the nuclear export of Net1A also remain to be defined. Schmidt and Hall showed that Net1 lacking most of the N-terminus (Net1ΔN) was exported from the nucleus in a CRM1 (Chromosome Region of Maintenance 1)-dependent manner.[18] Surprisingly, Net1 does not contain an identifiable nuclear export signal (NES) sequence. Instead, the PH domain mediates CRM1-dependent export of Net1ΔN, suggesting that this domain interacts with an NES-containing protein.[18] The identity of this protein remains unknown.

Our findings may have relevance to the control of other Rho regulatory enzymes. For example, the Net1-related protein XPLN (ARHGEF3) localizes to the nucleus in untreated U937 cells and relocalizes to the cytoplasm when treated with an HDAC inhibitor (MS275 or entinostat).[23] Moreover, the nuclear RhoGEF Epithelial cell transforming sequence 2 can relocalize to the cytoplasm to promote cell motility and invadopodia formation, although the mechanisms accounting for this relocalization are unknown.[24,25] In addition, the ability of RhoGDIα to bind RhoA is inhibited by lysine acetylation, leading to activation of RhoA and F-actin accumulation.[22]

It is perhaps not surprising that the activity of Rho regulatory proteins is controlled by acetylation, as acetylation is an important regulatory mechanism for other

cytoskeletal elements (Fig. 2). For example, acetylation of actin on its N-terminus may affect its affinity for myosin,[26] and mass spectrometry approaches have identified multiple lysines acetylated within actin monomers.[27] Similarly, cardiac myosin heavy chain motor activity is increased by lysine acetylation.[28] Acetylation of the actin binding protein cortactin regulates its actin binding function as well as its subcellular localization.[29-35] Moreover, acetylation of microtubules is correlated with increased stability, and deacetylation by HDAC6 and SIRT2 is necessary for cell motility.[29,36-40] Microtubule acetylation also improves its function as a scaffold for cargo-carrying kinesins.[41,42]

In summary, recent studies indicate that lysine acetylation of multiple proteins regulates actin cytoskeletal dynamics, microtubule function and cell motility. Undoubtedly, additional cytoskeletal regulatory proteins will be shown to be regulated by acetylation in the future. Our identification of the role of acetylation in controlling the subcellular localization of Net1A adds yet another layer to this regulatory theme. A better understanding of these regulatory mechanisms may enable the creation of better targeted therapies for diseases typified by altered cell motility, such as cancer metastasis.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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