Lysine-acetylation as a fundamental regulator of Ran function: Implications for signaling of proteins of the Ras-superfamily

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The small GTP-binding protein Ran is involved in the regulation of essential cellular processes in interphase but also in mitotic cells: Ran controls the nucleocytoplasmic transport of proteins and RNA, it regulates mitotic spindle formation and nuclear envelope assembly. Deregulations in Ran dependent processes were implicated in the development of severe diseases such as cancer and neurodegenerative disorders. To understand how Ran-function is regulated is therefore of highest importance. Recently, several lysine-acetylation sites in Ran were identified by quantitative mass-spectrometry, some being located in highly important regions such as the P-loop, switch I, switch II and the G5/SAK motif. We recently reported that lysine-acetylation regulates nearly all aspects of Ran-function such as RCC1 catalyzed nucleotide exchange, intrinsic nucleotide hydrolysis, its interaction with NTF2 and the formation of import- and export-complexes. As a hint for its biological importance, we identified Ran-specific lysine-deacetylases (KDACs) and -acetyltransferases (KATs). Also for other small GTPases such as Ras, Rho, Cdc42, and for many effectors and regulators thereof, lysine-acetylation sites were discovered. However, the functional impact of lysine-acetylation as a regulator of protein function has only been marginally investigated so far. We will discuss recent findings of lysine-acetylation as a novel modification to regulate Ras-protein signaling.

The Ran-Protein is Structurally and Functionally Distinct From Other Ras-Proteins: Lysine-Acetylation as a Specific Regulator of Ran-Function?

The progress in quantitative proteomics showed that lysine-acetylation is conserved from bacteria to man and enabled the identification of thousands of acetylation sites in all cellular compartments covering all essential cellular functions.1-7 Many acetylation sites were identified in Ras-related proteins as well as regulators and effectors thereof.

Ras-related proteins have a common structural motif, known as the G-domain.8 The Arf/Arl-related proteins have an N-terminal extension that can be myristoylated for membrane attachment, the Rho-proteins have an additional α-helix between β5 and α4, known as the insert region/helix.9 Ran carries an additional C-terminal helix, the C-terminal switch, followed by an acidic tail (211-DEDDDL-216). In the active state, it becomes detached from the G-domain enabling the interaction to effector proteins such as RanBP1, RanBP2 and importin β. In contrast to other Ras-proteins, upon GTP-hydrolysis, the switch I in Ran (and Arf) forms an additional highly ordered intramolecular β-sheet with the interswitch composed of β2 and β3.10-12

Many Ras-proteins are targeted to subcellular membranes by a C-terminally localized poly-basic patch (classical Rho-proteins, K-Ras4A) and/or by post-translational lipidation.13 This lipidation
includes the formation of stable thioethers by prenylation (geranylgeranyl: classical Rho proteins, Rab, Rap; farnesyl: Ras, Rnd1-3, TC10, TCL, RhoD, Rif) and/or by reversible fatty acid esterification such as palmitoylation (H-Ras, N-Ras, K-Ras4B, RhoB, Cdc42 isoform1, TC10) or myristoylation (Arf/Arli). Comparing the primary sequences of Ras-related proteins shows that the C-terminus is the most divergent part. Ran contains neither a C-terminally poly-basic patch nor motifs for lipidation. Although for some Ras-proteins a nuclear localization has been reported, they are predominantly cytosolic either attached to subcellular membranes or solubilized in the cytosol by binding to solubilizing factors such as RhoGDI, RabGDI or PDE6. In contrast, Ran was found to be more than 90% nuclear in interphase cells.

Notably, all mammalian species have multiple Ras-related, Rho-related, Arf-like and Rap-proteins. However, they contain only a single Ran gene, encoding for a single Ran-protein, being the most abundant protein of the Ras-superfamily in the cell. Notably, the existence of a variety of import- and export-receptors is a specificity determinant for the transport of different cargoes, at least to some extent.

In interphase cells, Ran regulates the directed exchange of proteins and RNA between nucleus and cytosol. Furthermore, Ran is an essential regulator during mitosis controlling the assembly of the nuclear envelope. Considering that the cell has just one single Ran variant a defect would have drastical consequences on cellular function. However, the cell can thereby ensure that those processes are tightly controlled since no other protein can complement Ran-function. Deregulations in Ran dependent processes were implicated in the development of severe diseases, such as cancer and neuro-degenerative disorders. To understand how Ran is regulated is therefore of highest importance. One way cells can modulate and specify protein-functionalities are post-translational modifications (PTMs). However, while for nearly all Ras-related proteins, PTMs such as phosphorylation, lipidation, and ubiquitylation are reported, none of these regulatory mechanisms were functionally described for Ran so far. However, recent progress in quantitative mass-spectrometry revealed that Ran is extensively targeted by lysine-acetylation. Lysine-acetylation is tightly connected to metabolism in that acetyl-CoA serves as the acetyl-donor molecule for lysine-acetyltransferases (KATs) and NAD+ is an obligatory co-factor of sirtuin (Sirt; silent information regulator)-deacetylases.

However, until recently, it was not clear how lysine-acetylation of Ran affects its function or how it is regulated.

**Ran-Function is Controlled by Lysine-Acetylation**

Several mass-spectrometry based screens enabled the identification of a total of 11 lysine-acetylation sites in Ran. Some of those sites are located in functionally highly important regions such as the P-loop, switch I and switch II as well as the G5/SAK-motif needed for nucleotide binding (K23R, K37R, K71R, K152R; superscript R: Ran) (Fig. 1). We used a synthetic biological approach to site-specifically introduce acetyl-L-lysine at the 5 sites in the Ran-protein originally identified by Choudhary and co-workers in 2009 (K37, K60, K71, K99, K152). Using a synthetically evolved acetyl-CoA synthetase/rRNA CUAP pair from Methanosarcina barkeri we functionally investigated how lysine-acetylation controls Ran-function (Fig. 1A). We discovered that the acetylations at K37R, K99R and K159R increase the affinity toward import- and export-receptors supporting import- cargo release and export-complex formation in the nucleus. Ran AcK99 has a loss-of-function phenotype lowering RCC1 affinity and the RCC1 catalyzed nucleotide exchange. Furthermore, Ran K99R shows a more cytosolic distribution in HeLa cells by an NTF2 independent mechanism as AcK99R and K99R both showed no influence on NTF2 binding. The acetylation of K71R has a dominant-negative effect by increasing the affinity toward RCC1 and lowering the the RCC1 catalyzed nucleotide exchange activity. Furthermore, it abolishes NTF2 binding leading to a cytosolic accumulation of the Ran-GDP-protein, which, as a consequence, would result in a block of nucleocytoplasmic transport. Mutation of Lys to Arg is often used to preserve a non-acetylatable state in cells to phenotypically characterize an acetylation event in comparison to a Lys to Gln mutation mimicking the acetylated state. We observed a nearly complete cytosolic Ran localization expressing K71Q, mimicking the AcK71R. Notably, K71R showed a markedly reduced NTF2-binding compared to RanWT. These results show, that these mutational approaches are sometimes misleading as Gln is not a perfect molecular mimic for an acetylated-lysine and Arg sometimes mimics an acetyl-lysine even better if exerting steric rather than electrostatic effects.

Using the genetic-code expansion concept allows to study the real impact of lysine-acetylation on protein-function.

**Lysine-acetylation is tightly connected to the cellular metabolic and energetic state.** The writers, lysine acetyltransferases (KATs), using acetyl-CoA as acetyl donor molecule and the erasers, sirtuin deacetylases, using NAD+ as essential cofactors for catalysis, directly connect lysine-acetylation to the cellular metabolism. We identified the KATs CBP, p300, Tip60 and αTAT as potential Ran-specific acetyl-transferases in in vitro and in vivo assays. Importantly, in contrast to published proteomic screens, we identified K37R, K134R, K142R and K152R as being lysine-acetylated using this combined in vitro and in vivo approach. This does of course not exclude that under specific cellular conditions also other Ran-lysines are acetylated in an enzymatic fashion or that Ran is acetylated by transferases not tested in our study. However, another possibility is that other acetylation sites occur non-enzymatically or at very low stoichiometries. In an in vitro screen for Ran lysine-deacetylases, we found that only 2 sites are deacetylated (AcK37 by

**Implications of Ran-Acetylation and Therapeutic Implications of Ran-Acetylation to Design Sirtuin Inhibitors**

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Sirt1, -2, and -3 and AcK71 only by Sirt2). Again, this does not exclude the existence of other decetylases catalyzing the deacetylation of acetylated Ran also on the other sites. It is known that KATs and KDACs are often active in multi-protein complexes, that their activities are regulated by post-translational modifications and even by binding to regulators such as fatty acids. Notably, we observed that the deacetylation at Ran AcK71 is faster in its active state, suggesting that structural features are important for the deacetylation by Sirt2. Based on these results, we are investigating the potency of Ran AcK71-derived peptides as Sirt2 inhibitors, which we hope can ultimately be further developed into therapeutics for the treatment of neurodegenerative diseases. In fact, peptide-based molecules are among the most potent and specific Sirt2 inhibitors identified so far.
Physiological Relevance of Ran-Lysine-Acetylation

Although our data demonstrate the strong impact lysine-acetylation has on Ran protein-function, the question remains under which conditions it is physiologically most significant. Lysine-acetylation might either occur enzymatically by KATs or, under certain conditions, non-enzymatically. Although it was shown that, in contrast to the mitochondrial matrix, the nucleus and in the cytosol do not allow for non-enzymatic acetylation to occur to a large extent, high acetyl-CoA levels or events increasing the nucleophilicity at the lysine’s-epsilon amino group could favor non-enzymatic lysine-acetylation. The latter includes factors that lower the lysine’s pK_A value such as a more basic pH or the presence of basic residues in vicinity. A lysine-acetylation site can only be of biological significance if it accumulates to sufficiently high stoichiometries unless it creates a gain-of-function for which a subpopulation of protein might be enough to mediate an effect. Alternatively, acetylation could also have additive effects if it occurs substoichiometrically in a pathway of multiple consecutive steps or if it influences the cooperativity of multi-subunit proteins. For Ran, the only acetylation site that could substoichiometrically exert a biological function is Ran AcK71 since it has a dominant-negative effect on RCC1 catalyzed nucleotide exchange. For the other effects we observed (of Ran AcK71 on nuclear localization of Ran-GDP or of AcK37, 99 and 159 on import and export complex formation) the acetylation needs to reach high stoichiometries to fulfill a biological role. Some Ran-acetylation sites such as AcK71 and AcK99 are downregulating Ran-function, whereas others such as AcK37, 99, 159 upregulate Ran-function in processes such as import cargo release in the nucleus and export-complex formation. Importantly, several additional lysine-acetylation sites were found in proteomic screens. Acetylation of K23^R (K16 in Ras) in the G1/P-loop and K152^R (K147 in Ras) in the G5/SAK-motif most likely interferes with nucleotide binding. K134^R in Ran makes interactions to the export receptor Crm1 and to the nucleotide release factor Mog1. For the basic-patch encompassing K141^R and K142^R, it was shown by a mutational approach that it affects Crm1, importin β and RanBP1 binding. How lysine-acetylation at these sites interferes with those interactions and how they are regulated by KATs and KDACs needs further investigation. Except from K23^R (K16 in Ras) in the P-loop and K152^R (K147 in Ras) in the G5/SAK motif, all the other acetylation sites show a weak conservation between members of the Ras-superfamily, albeit being located in functionally highly important regions. This suggests that this global regulation of Ran-function by lysine-acetylation is Ran-specific (Fig. 1D; Table 1). Interestingly, for many sites where a lysine is found in Ran there are negatively charged residues Glu or Asp at the homologous positions in other Ras-proteins, maybe indicating that the charge is of functional importance (Table 1). The exact physiological conditions under which Ran-acetylation is functionally important might very much depend on the cellular metabolic state. Notably, lysine-acetylation might play important roles in precisely defined physiological or cell cycle states not only in interphase but also in mitotic cells. For Ran-function lysine-acetylation is an important regulator.

### Lysine-Acetylation as a Regulator in Ras-Signaling

Although many acetylation sites were identified in Ras-proteins, effectors and regulators thereof, by quantitative proteomics, the functional consequences of these modifications are mostly unknown. Comparing acetylation sites in Ras-proteins identified in human cells, in mice and rat

### Table 1. Comparison of the amino-acids present at analogous positions in selected Ras-proteins representing the 5 major Ras-subfamilies to Ran lysine-residues identified as being lysine-acetylated. In cases where no residue is shown, at those positions there is the same residue as shown above

| GNBP   | 23 | 37 | 38 | 60 | 71 | 99 | 134 | 141 | 142 | 152 | 159 |
|--------|----|----|----|----|----|----|-----|-----|-----|-----|-----|
| Ran    | K  | K  | K  | K  | K  | K  | K   | K   | K   | K   | K   |
| K-Ras  | D  | E  | L  | E  | E  | E  | Y   | G   | D   |     |     |
| N-Ras  | A  | E  | R  |     |     |     |     |     |     |     |     |
| H-Ras  | E  | R  |     |     |     |     |     |     |     |     |     |
| Rap1A  | E  | K  | M  | Q  | N  | E  | W   | C   | E   |     |     |
| Rap1B  | V  | E  | D  | E  |     |     |     | I   | G   |     |     |
| RhoA   |     | D  | Q  |     |     |     |     |     |     |     |     |
| RhoB   | E  | S  |     |     |     |     |     |     |     |     |     |
| RhoC   |     |     |     |     |     |     |     |     |     |     |     |
| Cdc42  | S  | E  | T  |     | L  |     | K   | N   |     |     |     |
| Rac1   | G  | N  | P  | I  | G  | L   |     |     |     |     |     |
| Rab1A  | E  | S  | K  | R  | N  | Y   | S   | L   | K   | Q   |     |
| Rab1B  |     |     |     |     |     |     |     |     |     |     |     |
| Rab7A  | N  | Q  | T  | K   | K   | K   | N   |     |     |     |     |
| Rab7B  | E  | E  | K  | E   | E   |     | E   | D   |     |     |     |
| Rab7L  | K  | H  | R  |     | S   | D   | N   | G   |     |     | E   |
| Arf1   | T  | T  | K   | S   | G   | S   | R   | K   | T   |     |     |
| Arf1   | S  | K  | N   |     |     |     |     |     |     |     | N   |

192 Small GTPases Volume 6 Issue 4
Table 2. Lysine-residues in Ras-proteins representing the 5 major Ras-subfamilies identified to be lysine-acetylated by quantitative proteomics as found in PhosphoSitePlus. The sites in human were found in AS49 (pulmonary), HeLa (cervical), Jurkat (T lymphocyte), K562 (erythroid) and/or MV4-11 (macrophage) cells. The sites in mouse were identified in liver tissue and in the ones in rat were found in 11 different tissues (brain, heart, kidney, liver, lung, pancreas, skin, spleen, stomach, testis, thymus).

| Protein | Human | Mouse | Rat |
|---------|-------|-------|-----|
| Ran     | K37,60,71,99, 152,159 | K60,71,159 | K23,28,37,60,99, 134,152,159 |
| K-Ras   | K104  | —     | —   |
| N-Ras   | —     | —     | —   |
| H-Ras   | —     | —     | —   |
| Rap1A   | —     | —     | K151 |
| Rap1B   | —     | —     | K151 |
| RhoA    | —     | —     | K133,135 |
| RhoB    | —     | —     | —   |
| RhoC    | —     | —     | —   |
| Cdc42   | K135,144,153 | —     | K128,133,135, 144,150,153,166 |
| Rac1    | —     | K132, K133 | K147,153 |
| Rab1A   | K61   | —     | K61,132,140 |
| Rab1B   | K58   | —     | —   |
| Rab7A   | —     | —     | K32  |
| Rab7B   | —     | —     | —   |
| Rab7L   | —     | —     | —   |
| Af1     | —     | —     | K152 |
| Af1     | K36,142 | K142  | K36,142 |

Conclusions and Future Directions

Lysine-acetylation is tightly connected to the cellular metabolism. For Ras, this represents a regulatory system to adapt processes such as nucleocytoplasmic protein transport or the mitotic spindle formation to the cellular energetic state. Future studies will show to what extent acylations occur on lysines, if they occur simultaneously on distinct subpools and how the acylations, particularly of negatively charged acylations such as succinylation, exert mechanistically different functions. Particularly, poly-basic regions of Ras-proteins, due to their increased reactivity, might likely be targeted by these post-translational modifications.

However, those studies were only performed with the Gln and Arg mutants, used as a mimic for lysine-acetylation or to conserve the non-acetylated state, respectively. This Ras acetylation was shown to be counterbalanced by the cytosolic deacetylases Sirt2 and HDAC6. If this occurs directly or indirectly is not known. Future studies will show if acetylation has the same impact on SOS-catalyzed nucleotide exchange on K-Ras 4B and if K-Ras 4B is a direct Sirt2/HDAC6 target. For the Rho-nucleotide exchange factor Net1A, a recent study shows that this is mediated by lysine-acetylation within Net1A NLS-sequences. Also for Cdc42 and RhoA acetylation sites have been found in the insert-helix. In RhoGDIα, lysine-acetylation sites were found in its immunoglobulin domain as well as its N-terminal domain suggesting to interfere with the Rho-protein’s GDP/GTP and/or membrane/cytosol cycle. In the Rho-effector protein mDia1, lysine-acetylation sites have been identified in functionally highly important regions such as the Formin-homology 2 domain, essential for its actin nucleation capability.
methods need further technical improvement.\textsuperscript{2,47} Moreover, to show which of the sites are enzymatically acetylated, future studies should address which KATs have a robust lysine-acetyltransferase activity. Due to its low sequence homology, the identification of KATs is challenging. A recent report suggested that, apart from the classical KATs, there are additional orphan-KATs with putative lysine-acetyltransferase activity.\textsuperscript{48} Future studies are needed to show how KATs are regulated, how they are subcellually localized and what their substrates are.

As a summary, lysine-acetylation can represent a dynamic and powerful post-translational modification to control protein function far beyond just neutralizing a positive-charge. The identification of differently charged or uncharged acylations of different acyl-chain lengths adds another level of complexity on this post-translational modification and will be interesting to see how these acylations exert mechanistically different regulatory functions. Tackling the lysine-acetylation machinery might allow the development of novel therapeutic strategies in Ras-signaling.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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